IN VITRO CHARACTERIZATION OF LAMOTRIGINE N2-GLUCURONIDATION AND THE LAMOTRIGINE-VALPROIC ACID INTERACTION

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ABSTRACT:

Studies were performed to investigate the UDP-glucuronosyltransferase enzyme(s) responsible for the human liver microsomal N2-glucuronidation of the anticonvulsant drug lamotrigine (LTG) and the mechanistic basis for the LTG-valproic acid (VPA) interaction in vivo. LTG N2-glucuronidation by microsomes from five livers exhibited atypical kinetics, best described by a model comprising the expressions for the Hill (1869 ± 1286 μM, n = 0.65 ± 0.16) and Michaelis-Menten (Km = 2234 ± 774 μM) equations. The UGT1A4 inhibitor hecogenin abolished the Michaelis-Menten component, without affecting the Hill component. LTG N2-glucuronidation by recombinant UGT1A4 exhibited Michaelis-Menten kinetics, with a Km of 1558 μM. Although recombinant UGT2B7 exhibited only low activity toward LTG, inhibition by zidovudine and fluconazole and activation by bovine serum albumin (BSA) (2%) strongly suggested that this enzyme was responsible for the Hill component of microsomal LTG N2-glucuronidation. VPA (10 mM) abolished the Hill component of microsomal LTG N2-glucuronidation, without affecting the Michaelis-Menten component or UGT1A4-catalyzed LTG metabolism. Ks values for inhibition of the Hill component of LTG N2-glucuronidation by VPA were 2465 ± 370 μM and 387 ± 12 μM in the absence and presence, respectively, of BSA (2%). Consistent with published data for the effect of fluconazole on zidovudine glucuronidation by human liver microsomal UGT2B7, the Ks value generated in the presence of BSA predicted the magnitude of the LTG-VPA interaction reported in vivo. These data indicate that UGT2B7 and UGT1A4 are responsible for the Hill and Michaelis-Menten components, respectively, of microsomal LTG N2-glucuronidation, and the LTG-VPA interaction in vivo arises from inhibition of UGT2B7.

Lamotrigine [3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine; LTG] (Fig. 1) is an anticonvulsant with proven efficacy in the treatment of epilepsy (Pellock, 1994). It is one of a few agents approved for the treatment of both partial and generalized seizures, and is well tolerated (Vinik, 2005). Hepatic metabolism is the primary route of elimination for LTG, predominantly via N-glucuronidation (Cohen et al., 1987; Mikati et al., 1989). After an oral dose of LTG, approximately 85% is recovered in urine as a quaternary glucuronide at the N-2 position of the triazine ring (Fig. 1). The remainder is eliminated as the N5 glucuronide, an N2-methylated derivative, unidentified metabolites, and unchanged drug (Sinz and Remmel, 1991; Doig and Clare, 1991).

Glucuronidation reactions are catalyzed by the enzyme UDP-glucuronosyltransferase (UGT). UGT exists as a superfamily of enzymes that exhibit distinct, but overlapping, substrate and inhibitor selectivities (Miners et al., 2004; Kiang et al., 2005; Mackenzie et al., 2005). Seventeen human UGT proteins have been identified to date, and these have been classified into two families (UGT1 and UGT2) based on sequence identity (Mackenzie et al., 2005). Although the disposition of LTG is well characterized in vivo, the identity of the human hepatic UGT enzyme(s) responsible for the elimination of this drug has not been explored in a systematic manner. Green and Tephly (1996) reported that UGT1A4 glucuronidated LTG. In contrast UGT1A3, another enzyme known to glucuronidate amines, lacked LTG N2-glucuronidation activity. Given the broad role of UGT1A4 in amine glucuronidation, it is widely assumed that this enzyme is the principal catalyst of LTG N2-glucuronidation. However, the relative contribution of UGT1A4 to human liver microsomal LTG N2-glucuronidation at therapeutic concentrations is unknown.

Although LTG monotherapy has been shown to be effective in the treatment of epilepsy, this drug is more commonly administered in combination with other anticonvulsants (Hirsch et al., 2004). Thus, the potential exists for inhibitory drug-drug interactions. Indeed, co-administration of valproic acid (VPA), another anticonvulsant that is primarily eliminated by glucuronidation (Cotariu and Zaidman, 1988),...
is known to increase the area under the plasma-concentration time curve (AUC) for LTG in a dose-dependent manner in humans (Morris et al., 2000).

Recent work in this and other laboratories has explored in vitro approaches for predicting the hepatic clearance of glucuronidated drugs in vivo and the magnitude of inhibitory drug interactions involving glucuronidated drugs. In general, kinetic data generated using human liver microsomes (HLMs) as the enzyme source underestimate both hepatic clearance (CLH) and the magnitude of in vivo interactions for glucuronidated drugs (Boase and Miners, 2002; Soars et al., 2002). It has been observed, however, that the addition of bovine serum albumin (BSA) (2%) to incubations results in an increased in vitro CLH for zidovudine and a decreased Km for fluncozaole inhibition of zidovudine metabolism, such that the magnitude of the inhibitory interaction in vivo was predicted correctly (Uchaipichat et al., 2006b). The mechanism by which BSA decreases the Km of zidovudine and the Km of fluncozaole is unknown but is independent of drug and inhibitor protein binding. This result is in agreement with data published for CYP2C9 substrates, which show that the addition of BSA improves the predictive capacity of models used to predict the clearance of several drugs (Laddén et al., 1997; Carlile et al., 1999; Tang et al., 2002).

There is emerging evidence that UGTs other than UGT1A4 catalyze N-glucuronidation reactions. For example, it has been demonstrated recently that UGT2B7 mediates the N-glucuronidation of carbamazepine and BMS-204352 (Staines et al., 2004; Zhang et al., 2002). It has been observed, however, that the addition of bovine serum albumin (BSA) (2%) to incubations results in an increased in vitro CLH for zidovudine and a decreased Km for fluncozaole inhibition of zidovudine metabolism, such that the magnitude of the inhibitory interaction in vivo was predicted correctly (Uchaipichat et al., 2006b). The mechanism by which BSA decreases the Km of zidovudine and the Km of fluncozaole is unknown but is independent of drug and inhibitor protein binding. This result is in agreement with data published for CYP2C9 substrates, which show that the addition of BSA improves the predictive capacity of models used to predict the clearance of several drugs (Laddén et al., 1997; Carlile et al., 1999; Tang et al., 2002).

Materials and Methods

Materials. Alamethicin (from Trichoderma viride), BSA (fraction V, 98–99% protein), hecogenin (Hec), 4-methylumbelliferone (4MU), 4-methylumbelliferone β-D-glucuronide (4MUG), UDP-glucuronic acid (trisodium salt), and VPA were purchased from Sigma-Aldrich (Sydney, Australia). Lamotrigine (LTG) and lamotrigine N2-glucuronide (LTG-Gluc) were obtained from Wellcome Research Laboratories (Beckenham, UK). Solvents and other reagents were of analytical reagent grade.

Methods. Human Liver Microsomes and Expressed UGT Protein. Liver tissue (H07, H10, H12, H13, and H40) was obtained from the human liver “bank” of the Department of Clinical Pharmacology of Flinders University. Approval for the use of human liver tissue in xenobiotic metabolism studies was obtained from both the Clinical Investigation Committee of Flinders Medical Centre and from the donors’ next-of-kin. HLMs were prepared by differential centrifugation, as described by Bowalgaha et al. (2005).
incubation time was increased to 75 min. Incubations containing BSA (2%) were terminated by the addition of perchloric acid (6 μl, 70% v/v solution). Under the reaction conditions used, LGT N2-glucuronidation was linear with respect to incubation time to 75 min and protein concentration to 1.5 mg/ml with both HLMs and HEK293 cell lysate as the enzyme source. LGT N2-glucuronidation by lysate from untransfected HEK293 cells was not detectable.

Quantification of LGT-Gluc Formation. HPLC was performed using an Agilent 1100 series instrument (Agilent Technologies, Sydney, Australia) fitted with a Zorbax Eclipse XBD-C8 analytical column (4.6 × 150 mm, 5 μm; Agilent Technologies). Analytes were separated by gradient elution at a flow rate of 1 ml/min. Initial conditions were 91% phosphate buffer (25 mM, pH 7.4), containing triethylamine (200 μl/l) (mobile phase A) and 9% acetonitrile (mobile phase B). These conditions were held for 3 min; then, the proportion of mobile phase B was increased to 18% over 4 min and held for 1 min. Finally, the proportion of mobile phase B was increased to 55% over 1 min. Column eluant was monitored at 210 nm. The retention time for VPA was 2.9 min. LTG and VPA concentrations in dialysis samples were determined by comparison of peak areas to those of a standard curve, in the respective concentration ranges 50 to 3000 μM and 300 to 1200 μM. Within-day variation was assessed by measuring LTG (50 and 1500 μM) or VPA (300 and 1200 μM) (n = 5 for each concentration) content in samples containing phosphate buffer (0.1 M, pH 7.4), HLMs (0.5 mg) in phosphate buffer (0.1 M, pH 7.4) or a mixture of BSA with HLMs in phosphate buffer (0.1 M, pH 7.4). Coefficients of variation were less than 5% in all cases.

Data Analysis. Kinetic constants for LTG glucuronidation by recombinant UGT1A4 were generated by fitting experimental data to the Michaelis-Menten equation, whereas kinetic constants for LTG glucuronidation by HLMs were obtained by fitting data to a hybrid Michaelis-Menten-Hill equation using EnzFitter (Biosoft, Cambridge, UK): 

\[ v = \frac{V_{\text{max}} \times [S]^n}{K_m + [S]} \]

where [S] is the substrate concentration, \( K_m \) is the Michaelis constant (substrate concentration at 0.5 \( V_{\text{max}} \)), \( S_0 \) is the concentration at 0.5 \( V_{\text{max}} \) is the Hill coefficient (which reflects the degree of sigmoidicity of the velocity versus substrate-concentration relationship), and \( V_{\text{max}} \) and \( V_{\text{max}} \) are the maximal velocities for the two enzymes. \( K_i \) values for VPA inhibition of LTG glucuronidation were determined by fitting experimental data to the expression for the competitive, noncompetitive, uncompetitive and mixed inhibition models using EnzFitter (Biosoft). Goodness of fit to kinetic and inhibition models was assessed by comparison of the F-statistic, \( r^2 \) values, parameter standard errors, and 95% confidence intervals. Kinetic constants are reported as the mean value ± standard deviation of the parameter estimate.

Statistical Analysis. Where appropriate, statistical analysis (Wilcoxon signed rank test) was performed using SPSS 10.0.5 (SPSS Inc., Chicago, IL). Values of p less than 0.05 were considered significant.

Quantitative Prediction of the VPA-LTG Interaction. The magnitude of VPA inhibition of LTG hepatic clearance was predicted based on the increase in the AUC ratio caused by the presence of the inhibitor, using the equation:

\[ \frac{\text{AUC}_{\text{inlet}}}{\text{AUC}} = \frac{1}{f_i \times \left( \frac{f_m}{1 + \left( I/K_i \right)} \right) + 1 - f_i \times f_m} \]

where \( K_i \) is the inhibition constant determined in vitro, \( f_i \) is the ratio of hepatic to total clearance expressed as a fraction, \( f_m \) is the ratio of metabolic clearance to total clearance expressed as a fraction, and \( I \) is the estimate of in vivo inhibitor concentration (Ito et al., 1998).

Different values of \( I \) were used to predict the magnitude of the interaction between LTG and VPA, based on approaches described by Ito et al. (1998, 2004). These were: maximum inhibitor concentration in plasma (\( I_{\text{max,pl}} \)), maximum unbound inhibitor concentration in plasma (\( I_{\text{max,unbound}} \)), average inhibitor concentration in plasma (\( I_{\text{ave,pl}} \)), average unbound inhibitor concentration in plasma (\( I_{\text{ave,unbound}} \)), and average and maximum hepatic input concentration given by the inhibitor concentration in the blood plus the inhibitor concentration from gastrointestinal absorption (\( I_{\text{inlet,ave}} \)). The latter parameter was calculated as:

\[ I_{\text{inlet,ave}} = I_{\text{max,pl}} + \frac{k_i \times \text{Dose} \times f_i}{Q_h} \]

where \( I_{\text{max,pl}} \) is the maximum inhibitor concentration in blood, \( k_i \) is the absorption rate constant, \( f_i \) is the fraction of the inhibitor dose absorbed from the gastrointestinal tract, and \( Q_h \) is liver blood flow. The dose was taken as 500 mg (3467 µmol), assuming that an “average” daily dose is 500 mg twice daily. It should be noted that increasing the dose to 1000 mg resulted in approximately a 9% increase in the AUC ratio (see Table 3) determined using the hepatic input concentration. \( I_{\text{max,pl}} \) was taken as the upper limit of the plasma VPA therapeutic range for the treatment of epilepsy; that is, 100 mg/l (693 µM) (Dutta et al., 2003). The lower limit of the VPA therapeutic range is 50 mg/l (347 µM). Thus, the mid-point of the therapeutic range (520 µM) was used as \( I_{\text{ave,pl}} \). The unbound values of \( I_{\text{inlet,ave}} \) and \( I_{\text{inlet,ave}} \) were calculated by multiplying the respective variable in vivo concentrations by the fraction of VPA
unbound in blood ($f_{u,VPA}$), which was taken as 0.1 (Anderson et al., 1994). Because VPA is essentially completely absorbed from the gastrointestinal tract, $f_a$ is 1.0 (Bressolle et al., 1994). The absorption rate constant ($k_a$) for VPA was assumed to be 0.1/h (Ito et al., 2004). LTG AUC data for patients administered different doses of VPA have been reported by Morris et al. (2000). A VPA dose of 1000 mg/day increased the LTG AUC by 160%. Thus, the in vivo LTG AUC ratio ($AUC_{v}/AUC$) was taken as 2.60.

**Results**

**Binding of LTG and VPA to HLMs, HEK293 Cell Lysate and BSA.** The binding of LTG and VPA was calculated as the concentration of drug in the buffer compartment divided by the concentration of drug in the protein compartment, and was expressed as the fraction unbound in incubations ($f_{u,inc}$). LTG and VPA binding to both HLMs and HEK293 cell lysate was negligible (<5%) across the concentration ranges investigated. Both drugs did, however, exhibit significant binding to BSA (2%). The binding of LTG to BSA was independent of LTG concentration, but VPA $f_{u,inc}$ increased with increasing VPA concentration. The mean ($\pm$ S.D.) $f_{u,inc}$ value for LTG binding to BSA was 0.65 $\pm$ 0.03, whereas for VPA, $f_{u,inc}$ ranged from 0.15 $\pm$ 0.04 (at 300 $\mu$M) to 0.35 $\pm$ 0.02 (at 1200 $\mu$M). Significant binding was also observed for both drugs with mixtures of BSA (2%) and each protein source. Mean $f_{u,inc}$ values for LTG binding to mixtures of BSA and HLMs and to BSA and HEK293 cell lysate were 0.49 $\pm$ 0.07 and 0.30 $\pm$ 0.01, respectively. Binding was independent of LTG concentration and, notably, was not additive with the binding of LTG to each source individually. In contrast, mean $f_a$ values for binding of VPA to mixtures of BSA and HLMs and BSA and HEK293 cell lysate were identical to those for binding to BSA alone, ranging from 0.15 $\pm$ 0.01 (at 300 $\mu$M) to 0.35 $\pm$ 0.03 (at 1200 $\mu$M). It was also demonstrated for both LTG and VPA that the presence of the alternate drug had no effect on binding under the conditions investigated. Where binding was observed (to HLMs or BSA), the concentration of drug added to incubation mixtures was corrected for binding in calculations of kinetic parameters.

**Kinetics of LTG N2-Glucuronidation by HLMs.** Although data were adequately fitted to the two-enzyme Michaelis-Menten equation, LTG N2-glucuronidation by HLMs in the presence and absence of BSA was best described by a hybrid model comprising the Michaelis-Menten and Hill (with $n < 1$, i.e., negative cooperativity) equations (Fig. 2); all goodness of fit parameters (see Data Analysis) were superior using the hybrid model. Derived kinetic parameters are shown in Table 1. In the absence of BSA, the mean $K_m$ value of the Michaelis-Menten component was 2234 $\pm$ 774 $\mu$M, whereas the mean $S_{50}$ and $n$ values of the Hill component were 1869 $\pm$ 1286 $\mu$M and 0.65 $\pm$ 0.16, respectively. The addition of BSA did not significantly affect the $K_m$ but did cause a significant ($p < 0.05$) decrease in
the mean $S_{50}$ (by approximately 65%) (Table 1). In the absence of BSA, the mean (±S.D.) $V_{\text{max}}$ values for the Michaelis-Menten and Hill components were 594 ± 333 and 162 ± 108 pmol/min · mg, respectively. BSA did not have a significant effect on the mean $V_{\text{max}}$ of either component, nor did it affect the Hill coefficient. It should be noted, however, that because the maximum unbound concentration of LTG in incubations conducted in the presence of BSA was approximately 1500 μM (i.e., maximum total concentration 3000 μM with $f_u$ 0.49), kinetic constants obtained from the experiments in the presence of albumin should be considered as estimates (although data for the Michaelis-Menten component are internally consistent).

Kinetics of LTG N2-Glucuronidation by HLMs in the Presence of Hecogenin. The highly selective UGT1A4 inhibitor Hec (Uchai-pichat et al., 2006a) was added to incubation samples (final concentration 10 μM) to confirm the involvement of this enzyme in human liver microsomal LTG N2-gluconidation. Derived kinetic parameters are shown for experiments performed in the presence and absence of BSA (2%) in Table 2. LTG-Gluc formation by HLMs in the presence of Hec, with and without 2% BSA, was described by the Hill equation with $n < 1$ (Fig. 2); that is, Hec abolished the Michaelis-Menten component of LTG N2-glucuronidation. In the absence of BSA, the mean (±S.D.) value of $S_{50}$ was 1848 ± 256 μM. For all livers, the addition of 2% BSA to incubation samples caused a significant ($p < 0.05$) reduction in the $S_{50}$ (by approximately 80%), and a relatively minor, but significant ($p < 0.05$), reduction in mean $V_{\text{max}}$ (approximately 30%). The addition of 2% BSA had no effect on the Hill coefficient.

LTG N2-Glucuronidation by Recombinant UGTs. Activities of UGT1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, 2B15, and 2B17 with 4MU as the substrate were similar to those reported by Uchai-pichat et al. (2004). 4MU glucuronidation activity of UGT 2B4 and 2B28 was confirmed using a radiometric thin-layer chromatographic method. UGT1A4 activity with trifluoperazine as the substrate was in agreement with previously reported data (Uchai-pichat et al., 2006a). Together with UGT2B10 and 2B28, which show very low or no activity toward all substrates tested to date, these enzymes were screened for LTG-N2-glucuronidation activity. At an LTG concentration of 1500 μM, UGT1A4 exhibited the highest activity (105 pmol/min · mg), whereas UGT1A1, UGT1A3, UGT1A6, UGT1A7, and UGT2B7 exhibited lesser activity (<6% that of UGT1A4).

Kinetics of LTG N2-Glucuronidation by Recombinant UGT1A4. LTG-Gluc formation by UGT1A4, in the presence and absence of 2% BSA, exhibited Michaelis-Menten kinetics (Fig. 3). In the absence of BSA, the $K_m$ and $V_{\text{max}}$ values for LTG-Gluc formation by UGT1A4 were 1558 μM and 224 pmol/min · mg, respectively. The addition of 2% BSA resulted in significant ($p < 0.05$) increases in both $K_m$ and $V_{\text{max}}$ (to 3235 μM and 1278 pmol/min · mg, respectively). As with previous experiments conducted in the presence of BSA, the maximum unbound concentration of LTG in incubations was approximately 1500 μM, and hence derived kinetic parameters should be considered as estimates. The addition of Hec (10 μM) to incubations performed with recombinant UGT1A4 decreased the rate of LTG-Gluc formation at substrate concentrations ranging from 10 to 3000 μM by approximately 95% (data not shown). The low rate of formation of LTG-Gluc by UGT1A1, 1A3, 1A6, 1A7, and 2B7 HEK293 cell lysates precluded full kinetic analysis.

Inhibition of LTG-Gluc Formation by UGT2B7 Substrates/Inhibitors. The known UGT2B7 substrate zidovudine (3 mM) (Court et al., 2003) and the selective UGT2B7 inhibitor fluconazole (2.5

### Table 1

Mean derived kinetic parameters for LTG N2-glucuronidation by human liver microsomes, with and without BSA

<table>
<thead>
<tr>
<th>Component</th>
<th>Without BSA</th>
<th>With BSA</th>
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<tr>
<td>$S_{50}$ (μM)</td>
<td>1869 ± 1286</td>
<td>255 ± 170</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (pmol/min/mg)</td>
<td>162 ± 108</td>
<td>99 ± 46</td>
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<tr>
<td>$n$</td>
<td>0.65 ± 0.16</td>
<td>0.59 ± 0.16</td>
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### Table 2

Mean derived kinetic parameters for LTG N2-glucuronidation by human liver microsomes in the presence of hecogenin, with and without BSA

<table>
<thead>
<tr>
<th>Component</th>
<th>Without BSA</th>
<th>With BSA</th>
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<tbody>
<tr>
<td>$S_{50}$ (μM)</td>
<td>1848 ± 256</td>
<td>382 ± 81</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (pmol/min/mg)</td>
<td>235 ± 29</td>
<td>156 ± 18</td>
</tr>
<tr>
<td>$n$</td>
<td>0.74 ± 0.04</td>
<td>0.71 ± 0.01</td>
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**FIG. 3.** Representative Eadie-Hofstee plot for LTG N2-glucuronidation by recombinant human UGT1A4, with and without BSA (2%). Units of $V/S$ are pmol glucuronide/μmol · min · mg. Points are experimentally determined values, whereas lines are from model-fitting.
mM) (Uchaipichat et al., 2006b) inhibited LTG-Gluc formation by pooled HLMs at an LTG concentration of 25 μM by 70% and 74%, respectively, and by 22% and 20%, respectively, at an LTG concentration of 1500 μM (data not shown).

**VPA Inhibition of LTG-Gluc Formation Catalyzed by HLMs and Recombinant UGT1A4.** Inhibition of UGT1A4-catalyzed LTG N2-glucuronidation by VPA was investigated in the presence and absence of 2% BSA. VPA caused <5% inhibition of LTG-Gluc formation by recombinant UGT1A4, irrespective of the presence of BSA. Moreover, VPA (10 mM) added to incubations of pooled HLMs essentially abolished the Hill (i.e., non-UGT1A4) component of LTG N2-glucuronidation over the substrate concentration range 25 to 3000 μM. Kinetic data generated in the presence of VPA (10 mM) were well described by the Michaelis-Menten equation (data not shown); derived $K_m$ (1309 μM) and $V_{max}$ (686 pmol/min·mg) were within the ranges obtained for the Michaelis-Menten component (i.e., UGT1A4) of the LTG N2-glucuronidation by microsomes from individual livers in the absence of BSA (Table 1). Taken together, these observations indicate that VPA has no effect on UGT1A4-catalyzed LTG N2-glucuronidation. Hec (10 μM) was therefore added to incubations to remove the UGT1A4 component of the microsomal reaction. For incubations conducted in the presence of Hec, VPA inhibited LTG N2-glucuronidation by HLMs in a competitive manner in the presence and absence of BSA (Fig. 4). The addition of 2% BSA to incubation samples caused a significant ($p < 0.05$) reduction in the mean $K_i$ determined with microsomes from livers H7, H10, H12, H13, and H40, from 2465 ± 370 to 387 ± 12 μM. The addition of 2% BSA did not alter the model (i.e., competitive) of inhibition. As noted previously, correction of the binding of LTG and VPA to BSA (and other incubation components) was accounted for in the calculation of $K_i$ values.

**Prediction of in Vivo AUC Ratio.** Predicted LTG AUC ratios in vivo (in the presence and absence of coadministered VPA) based on various value of $[I]$ (see Materials and Methods) are shown in Table 3 for $K_i$ values generated in both the absence and presence of BSA. Predicted AUC ratios calculated using $K_i$ values generated in the absence of BSA ranged from 1.00 to 1.28. The use of $K_i$ values generated in the presence of BSA resulted in higher predicted AUC ratios (1.01–2.31). However, only calculations that incorporated the total VPA (inhibitor) concentration predicted a clinically significant interaction (predicted AUC ratio 1.86–2.31).

**Discussion**

The microsomal kinetic studies reported here are consistent with the involvement of at least two UGTs in LTG N2-glucuronidation. Kinetic data were well described by a hybrid model, comprising Michaelis-Menten and Hill components. Hec, a highly selective inhibitor of UGT1A4 (Uchaipichat et al., 2006a), abolished activity of the Michaelis-Menten component without affecting the kinetic parameters for the Hill component. The mean $K_m$ for the Michaelis-Menten component of LTG N2-glucuronidation by HLMs in the absence of Hec (viz. 2234 ± 774 μM) was similar in value to the $K_m$ for UGT1A4-catalyzed LTG N2-glucuronidation (1558 μM). Taken together, these data indicate that UGT1A4 is responsible for the Michaelis-Menten component of the microsomal reaction.

Apart from recombinant UGT1A4, UGT 1A1, 1A3, 1A6, 1A7, and 2B7 catalyzed LTG N2-glucuronidation, albeit at lower rates (3.1–5.4 pmol/min·mg versus 105 pmol/min·mg at a substrate concentration of 1500 μM). The low activities of these enzymes precluded full kinetic analysis. Substitution of the mean kinetic parameters for human liver microsomal LTG N2-glucuronidation in the hybrid Michaelis-Menten-Hill equation indicated that the contribution of the Hill component to the total activity was 58% and 20% at substrate concentrations of 25 μM and 1500 μM, respectively. Zidovudine (a selective substrate for UGT2B7; Court et al., 2003) and fluconazole (a selective inhibitor of UGT2B7; Uchaipichat et al., 2006b) inhibited human liver microsomal LTG N2-glucuronidation by approximately 70% at the lower substrate concentration, and approximately 20% at the higher concentration. Nevertheless, a role for UGT1A1, 1A3, and 1A6 (the other hepatically expressed enzymes shown here to form LTG-Gluc in the Hill component of human liver microsomal LTG N2-glucuronidation cannot be discounted. At present, selective inhibitors of these enzymes are unavailable. It should be noted that the involvement of UGT2B7 in LTG N2-glucuronidation is consistent with recent studies showing that this enzyme has the capacity to N-glucuronidate a number of drugs (Staines et al., 2004; Zhang et al., 2004). Interestingly, Staines et al. (2004) also reported very low activity ($V_{max} = 0.79$ pmol/min·mg) for carbamazepine N-glucuronidation by recombinant UGT2B7 with near complete inhibition of the human liver microsomal reaction by morphine and some other UGT2B7 substrates. Although this group suggested that carbamazepine and LTG were not glucuronidated by the same enzyme on the basis of minor inhibition of carbamazepine N-glucuronidation by LTG, the concentration of LTG used in the inhibition experiments (1000 μM) was low compared with the $S_{50}$ for the putative UGT2B7 component of LTG N2-glucuronidation reported here.

A recent study conducted in this laboratory (Uchaipichat et al., 2006b) demonstrated that BSA (2%) reduced the $K_m$ for zidovudine (a selective UGT2B7 substrate) glucuronidation by HLMs and recombinant UGT2B7 approximately 10-fold, without affecting $V_{max}$. Here, BSA (2%) reduced the $S_{50}$ for the Hill component (proposed to be
UGT2B7) of microsomal LTG N2-glucuronidation approximately 8-fold (without a significant effect on $V_{\text{max}}$ or the Hill coefficient), but did not alter the derived kinetic constants for the Michaelis-Menten component of the microsomal reaction. In contrast, addition of BSA to incubations increased both the $K_m$ and $V_{\text{max}}$ for LTG N2-glucuronidation by recombinant UGT1A4. The reason for the differing effects of BSA on human liver microsomal and recombinant UGT1A4 is currently unknown. However, Tang et al. (2002) proposed that BSA reduces the $K_m$ values of certain CYP2C9 substrates as a result of altered protein conformation or the “mopping up” of endogenous inhibitors present in microsomal incubations. Similar mechanisms may account for the effect of BSA on UGT2B7. A number of fatty acids are potent inhibitors of UGT2B7, with $K_i$ values as low as 0.15 $\mu$M (Tsoutsikos et al., 2004). Hence, the addition of exogenous albumin to microsomal incubations may bind inhibitory fatty acids, with a reduction in the “apparent” $K_m$ of the alternate substrate. It has also been reported recently that human serum albumin (HSA) was selected in yeast two-hybrid screening with UGT1A1 as the “bait” (Ohta et al., 2005). Furthermore, HSA bound to His-tagged UGT1A1 immobilized on a Ni-NTA resin, and an interaction between these proteins on the endoplasmic reticulum was hypothesized. Whether albumin interacts with UGT2B7 is currently under investigation in this laboratory. It should be noted that BSA and HSA both reduce the $K_m$ (and $K_i$) values of UGT2B7 substrates to a similar extent (A. Rowland, P. I. Mackenzie, and J.O. Miners, manuscript in preparation).

Coadministration of VPA is known to reduce LTG clearance in vivo in a dose-dependent manner. In this study, VPA inhibited the Hill component of microsomal LTG N2-glucuronidation, but not the Michaelis-Menten component. Consistent with the hypothesis that UGT1A4 represents the Michaelis-Menten component, VPA did not inhibit LTG N2-glucuronidation by recombinant UGT1A4, VPA is a known substrate of UGT2B7 (Jin et al., 1993; Ethell et al., 2003), and VPA has previously been shown to inhibit zidovudine glucuronidation by UGT2B7 (Trappnell et al., 1998; Ethell et al., 2003). For experiments performed in the presence of Hec, addition of BSA (2%) caused a 6-fold reduction in the $K_i$ for VPA inhibition of human liver microsomal LTG N2-glucuronidation, with no effect on the mechanism of inhibition (i.e., competitive). The reduction in $K_i$ was associated with a significant improvement in the prediction of the magnitude of the LTG-VPA interaction in vivo, as also observed by Uchaipichat et al. (2006b) when predicting the extent of the fluconazole-zidovudine interaction. Using the mean $K_i$ value generated in the presence of BSA and various estimates of total VPA concentration in vivo, the predicted LTG AUC ratio in the presence and absence of VPA ranged from 1.86 ($I/AUC$) to 2.31 ($I/AUC$). Morris et al. (2000) reported that the LTG AUC ratio increased, on average, by 2.6-fold in patients receiving VPA, 1000 mg/day (presumed to be 500 mg b.i.d.). In contrast, use of unbound VPA concentrations in the expression for the AUC ratio did not predict an interaction.

The nonhyperbolic kinetics for LTG N2-glucuronidation by HLMs observed here contrasts with a previous report of Michaelis-Menten kinetics ($K_m$ 2560 $\mu$M, $V_{\text{max}}$ 0.65 mmol/min · mg) for this pathway (Magdalou et al., 1992). Substrate concentration ranges differed between the two studies; 12 concentrations in the range 10 to 3000 $\mu$M in the present study versus 5 concentrations in the range 500 to 8000 $\mu$M in the previous work. To obtain substrate (LTG) concentrations above 3000 $\mu$M, the earlier study required a high content of DMSO (5% v/v) in microsomal incubations, and it is possible that this may selectively alter the activities of the microsomal UGTs that contribute to LTG N2-glucuronidation (Uchaipichat et al., 2004). Furthermore, transformation of the velocity versus substrate concentration data published by Magdalou et al. (1992) provides nonlinear Eadie-Hofstee plots inconsistent with Michaelis-Menten kinetics, suggesting that the original kinetic analysis may not have been appropriate.

In summary, both UGT1A4 and UGT2B7 appear to contribute to LTG N2-glucuronidation by human liver microsomes, with the UGT2B7-catalyzed reaction apparently dominating at low substrate concentrations. Given that the therapeutic plasma concentration range for LTG is 3 to 14 mg/l (12–55 $\mu$M), inhibition of UGT2B7-catalyzed LTG N2-glucuronidation provides a mechanism for the LTG-VPA interaction in vivo. VPA inhibits the UGT2B7, but not the UGT1A4, component of LTG N2-glucuronidation. Consistent with a recent report from this laboratory demonstrating that BSA reduced the $K_m$ for zidovudine glucuronidation by human liver microsomal UGT2B7, addition of BSA (2%) to incubations caused a 6-fold reduction in the $S_{50}$ for the putative UGT2B7 component of LTG N2-glucuronidation. Similarly, significant inhibition of LTG N2-glucuronidation by VPA in vivo was predicted from the $K_i$ value generated in the presence, but not the absence, of BSA. Together with the known effect of VPA on zidovudine glucuronidation both in vitro and in vivo (Lertora et al., 1994; Trappnell et al., 1998; Ethell et al., 2003), these data suggest that VPA may inhibit the metabolism of other substrates metabolized by UGT2B7, but not the glucuronidation of predominantly UGT1A4 substrates.

References


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