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***In vitro* characterization of lamotrigine N2-glucuronidation and the lamotrigine –
valproic acid interaction**

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Abbreviations:

AUC, area under the plasma concentration – time curve; BSA, bovine serum albumin; CYP, cytochrome P450; HLM, human liver microsomes; LTG, lamotrigine; LTG-Gluc, lamotrigine N2-glucuronide; 4-MU, 4-methylumbiliferone; 4-MUG, 4-methylumbiliferone- β -D-glucuronide; UDPGA, UDP-glucuronic acid, UGT, UDP-glucuronosyltransferase; VPA, valproic acid.

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Abstract

Studies were performed to investigate the UDP-glucuronosyltransferase enzyme(s) responsible for the human liver microsomal (HLM) 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 ($1869 \pm 1286\mu\text{M}$, $n\ 0.65 \pm 0.16$) and Michaelis-Menten ($K_m\ 2234 \pm 774\mu\text{M}$) 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 K_m of $1558\ \mu\text{M}$. Although recombinant UGT2B7 exhibited only low activity towards LTG, inhibition by zidovudine and fluconazole and activation by BSA (2%) strongly suggested that this enzyme was responsible for the Hill component of microsomal LTG N2-glucuronidation. VPA (10mM) abolished the Hill component of microsomal LTG N2-glucuronidation, without affecting the Michaelis-Menten component or UGT1A4-catalyzed LTG metabolism. K_i values for inhibition of the Hill component of LTG N2-glucuronidation by VPA were $2465 \pm 370\mu\text{M}$ and $387 \pm 12\mu\text{M}$ in the absence and presence of BSA (2%), respectively. Consistent with published data for the effect of fluconazole on zidovudine glucuronidation by human liver microsomal UGT2B7, the K_i 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 of microsomal LTG N2-glucuronidation, respectively, and the LTG – VPA interaction *in vivo* arises from inhibition of UGT2B7.

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Introduction

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 generalised seizures, and is well tolerated (Vinik *et al.* 2005). Hepatic metabolism is the primary route of elimination for LTG, predominantly via N-glucuronidation (Cohen *et al.* 1987; Mikati *et al.* 1989). Following 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 N-5 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 which exhibit distinct, but overlapping, substrate and inhibitor selectivities (Miners *et al.* 2004; Mackenzie *et al.* 2005; Kiang *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 characterised *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 *et al.* (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.

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While 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 HLM as the enzyme source underestimate both hepatic clearance (CL_H) 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* CL_{int} for zidovudine and a decreased K_i for fluconazole inhibition of zidovudine metabolism, such that the magnitude of the inhibitory interaction *in vivo* was predicted correctly (Uchaipichat *et al.* 2006a). The mechanism by which BSA decreases the K_m of zidovudine and the K_i of fluconazole is unknown, but is independent of drug and inhibitor protein binding. This result is in agreement with data published for cytochrome P4502C9 (CYP2C9) substrates, which show that the addition of BSA improves the predictive capacity of models used to predict the clearance of several drugs (Ludden *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.* 2004), and multiple UGT1A subfamily enzymes

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metabolise retigabine (Hiller *et al.* 1999). This work sought to characterize the kinetics of LTG glucuronidation by HLM and recombinant human UGTs and to assess the contribution of known UGTs to LTG hepatic clearance. In addition, the mechanism of the LTG – VPA interaction was investigated. Studies were performed with and without added BSA to determine whether *in vitro* data accurately predicted the magnitude of the LTG-VPA interaction, as has been recently reported for the fluconazole - zidovudine interaction.

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Materials and Methods

Materials

Alamethicin (from *Trichoderma viride*), bovine serum albumin (fraction V, 98-99% protein) (BSA), hecogenin (Hec), 4-methylumbelliferone (4MU), 4-methylumbelliferone β -D-glucuronide (4MUG), uridine-diphospho-glucuronic acid (UDPGA, trisodium salt) and valproic acid (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. Human liver microsomes (HLM) were prepared by differential centrifugation, as described by Bowalgaha *et al.* (2005).

cDNAs encoding UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, 2B17 and 2B28 were stably expressed in a human embryonic kidney cell line (HEK293), as described previously (Sorich *et al.* 2002; Stone *et al.* 2003; Uchaipichat *et al.* 2004). Cell lines were transfected separately with UGT cDNAs cloned into the expression vector pEF-IRES-puro6. The cells were incubated in Dulbecco's modified

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Eagle medium, which contained puromycin (1mg/L), fetal calf serum (10%), and penicillin G (100units/ml) – streptomycin (100mg/L) in a humidified incubator at 37°C with an atmosphere of 5% CO₂. Following growth to at least 80% confluency, cells were harvested and washed with phosphate buffered saline (0.1M, pH 7.4). Cells were subsequently lysed by sonication using a Heat Systems-Ultrasonics sonicator set at microtip limit of four. Cells expressing UGT1A enzymes were sonicated with four 'bursts' each lasting 2 sec, separated by 3 min with cooling on ice. Cells expressing UGT2B enzymes were treated using the same method, except sonication was limited to 1 sec bursts. Lysed samples were centrifuged at 12000g for 1 min at 4°C, and the supernatant fraction was removed and stored at –80°C until use. Expression of each UGT was demonstrated by immunoblotting with a commercial UGT1A antibody and a non-selective UGT antibody (raised against purified mouse Ugt) (Uchaipichat *et al.* 2004) and, where possible, activity measurements (see below).

Confirmation of UGT Activity

Activities of recombinant UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17 and 2B28 were confirmed using the non-selective substrate 4-methylumbelliferone (4MU). For all enzymes except UGT2B4 and UGT2B28 the conversion of 4MU to 4MUG was measured according to the spectrofluometric procedure of Miners *et al.* (1988), as modified by Uchaipichat *et al.* (2004). The concentration of 4MU present in incubations corresponded to the known K_m (or S₅₀) for each enzyme (Sorich *et al.* 2002; Uchaipichat *et al.* 2004). Given the lower specific activities of UGT 2B4 and 2B28, 4MU glucuronidation activity (at 1 mM) was confirmed using a radiometric thin layer chromatographic procedure (Jin *et al.*, 1997). UGT1A4 activity was confirmed using trifluoperazine as the substrate, at the

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approximate K_m for this substrate (40 μ M), according to the method of Uchaipichat *et al.* (2006b). The activity of UGT2B10 towards 4MU, and indeed most other substrates, is low or absent (Miners *et al.* 2004). Thus, it was not possible to include a positive control for this enzyme.

LTG N2-glucuronidation assay

Incubations, in a total volume of 200 μ l, contained MgCl₂ (4mM), HLM (0.1mg) and LTG (10-3000 μ M). The LTG was dissolved in 1M phosphoric acid containing 10% acetonitrile. Phosphate buffer (0.1M, pH 7.4) was generated in situ by the addition of KOH (1M, 37.2 μ l) to each incubation tube. LTG solutions were diluted 1:10 upon addition to incubations, such that the final concentration of acetonitrile was 1% v/v. This concentration of acetonitrile has a minor effect on UGT enzyme activities (Uchaipichat *et al.* 2004). Given the limited solubility of LTG, the maximal concentration possible in incubations was 3000 μ M. HLM were fully activated by the addition of the pore-forming polypeptide alamethicin (50 μ g/mg protein) with incubation on ice for 30 min (Boase and Miners, 2002). Following a 5 min pre-incubation, reactions were initiated by the addition of UDPGA (5mM). Incubations were performed at 37°C in a shaking water bath for 60 min. Reactions were terminated by the addition of perchloric acid (2 μ l, 70% v/v). Samples were subsequently centrifuged at 4000g for 10 min, and a 40 μ l aliquot of the supernatant fraction was injected directly into the HPLC column. For reactions performed using recombinant UGTs, incubation mixtures contained HEK293 cell lysate (0.2mg) in place of HLM protein, and the incubation time was increased to 75 min. Incubations containing BSA (2%) were terminated by the addition of perchloric acid (6 μ l, 70% v/v solution). Under the reaction conditions employed, LTG N2-glucuronidation was linear with respect to incubation time to 75

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min, and protein concentration to 1.5mg/ml with both HLM and HEK293 cell lysate as the enzyme source. LTG N2-glucuronidation by lysate from untransfected HEK293 cells was not detectable.

Quantification of LTG-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 x 150mm, 5 μ m; Agilent Technologies). Analytes were separated by gradient elution at a flow rate of 1ml/min. Initial conditions were 91% phosphate buffer (25mM, pH 7.4), containing triethylamine (200 μ L/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 1min. Finally, the proportion of mobile phase B was increased to 55% over 1 min. Column eluant was monitored by UV absorbance at 254nm. Retention times for LTG-Gluc and LTG were 5.3 and 10.3 min, respectively. LTG-Gluc formation was quantified by comparison of peak areas to those of an authentic LTG-Gluc standard curve prepared over the concentration range 1 – 20 μ M. The lower limit of quantification, defined as five times background absorbance, was 4 pmol/LTG-Gluc injected (corresponding to a rate of approximately 1 pmol/min.mg using recombinant UGTs as the enzyme source). Overall within day assay reproducibility was assessed by measuring LTG-Gluc formation in 8 separate incubations of the same batch of HLM (H40). Coefficients of variation were 4.1% and 2.4% for LTG concentrations of 100 μ M and 3000 μ M, respectively.

Inhibition of LTG-Gluc formation

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Incubations of both HLM and recombinant UGTs were performed in the absence and presence of hecogenin (Hec) and valproic acid (VPA). Hec (10 μ M), a known highly selective inhibitor of UGT1A4 (Uchaipichat *et al.* 2006b), was added to incubations to assess the contribution of this enzyme to LTG glucuronidation. VPA inhibition of LTG glucuronidation was investigated using microsomes from 5 human livers (H7, H10, H12, H13 and H40) and with UGT1A4 HEK293 cell lysate. Experiments performed to determine the inhibitor constant (K_i) for VPA inhibition of LTG glucuronidation included four VPA concentrations (300, 600, 900 and 1200 μ M) at each of three LTG concentrations (200, 400 and 600 μ M).

Binding of LTG and VPA to BSA, HLM and HEK293 cell lysate

Binding of LTG and VPA to HLM, HEK293 cell lysate, BSA (2%) and mixtures of BSA with each enzyme source was measured using an equilibrium dialysis method (McLure *et al.* 2000). Binding measurements were performed using a Dianorm equilibrium dialysis apparatus that comprised Teflon™ dialysis cells (capacity of 1200 μ l per side) separated into two compartments with Sigma Aldrich dialysis membrane (molecular weight cut off 12kDa). Membranes were conditioned overnight at 4°C in phosphate buffer (0.1M, pH 7.4). One side of the dialysis cell was loaded with 1ml of a solution of either LTG (12.5 to 3000 μ M) or VPA (300 to 1200 μ M) in phosphate buffer (0.1M, pH 7.4). The other compartment was loaded with 1ml of either a suspension of HLM (0.5mg) in phosphate buffer (0.1M, pH 7.4), HEK293 cell lysate (1mg) in phosphate buffer (0.1M, pH 7.4), BSA (2%) in phosphate buffer (0.1M pH 7.4), or a combination of BSA (2%) with each enzyme source in phosphate buffer (0.1M, pH 7.4). The dialysis cell assembly was immersed in a water bath maintained at 37°C and rotated at 12rpm for 5hr. Control experiments were also performed with phosphate buffer or HLM (0.25mg) on both sides of the dialysis cells at low and high

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concentrations of both drugs to ensure that equilibrium was attained. A 200 μ l aliquot was collected from each compartment, treated with ice-cold methanol containing 4% glacial acetic acid (200 μ l), and cooled on ice. Samples were subsequently centrifuged at 5000g for 10 min at 10°C and an aliquot of the supernatant fraction (5 μ l for LTG, 40 μ l for VPA) was analysed by HPLC.

Quantification of LTG and VPA binding

The HPLC system used was as described previously for LTG-Gluc formation. Separation of LTG was achieved using a 63:37 mixture of mobile phases A and B utilised for the LTG-Gluc assay. The mobile phase flow rate was 1.0ml/min and column temperature was 25°C. Column eluant was monitored at 254nm. The retention time for LTG was 2.6 min. VPA separation was achieved using an isocratic mobile phase comprising a 55:45 mixture of phosphate buffer (pH 3.1, 25mM) and acetonitrile at a flow rate of 1.0ml/min and a column temperature of 25°C. Column eluant was monitored at 210nm. 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 μ M and 300 to 1200 μ M. Within day variation was assessed by measuring LTG (50 and 1500 μ M) or VPA (300 and 1200 μ M) (n=5 for each concentration) content in samples containing phosphate buffer (0.1M, pH 7.4), HLM (0.5mg) in phosphate buffer (0.1M, pH 7.4) or a mixture of BSA with HLM in phosphate buffer (0.1M, pH 7.4). Coefficients of variation were less than 5% in all cases.

Data analysis

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

$$v = \frac{V_{max1} \times [S]^n}{S_{50}^n + [S]^n} + \frac{V_{max2} \times [S]}{K_m + [S]}$$

where [S] is the substrate concentration, K_m is the Michaelis constant (substrate concentration at $0.5 V_{max}$), S_{50} is the concentration at $0.5 V_{max}$, n is the Hill coefficient (which reflects the degree of sigmoidicity of the velocity versus substrate concentration relationship), and V_{max1} and V_{max2} 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, non-competitive, uncompetitive and mixed inhibition models using EnzFitter (Biosoft, Cambridge, UK). 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 \pm 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, USA). Values of p less than 0.05 were considered significant.

Quantitative prediction of the VPA – LTG interaction

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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:

$$\frac{AUC_{(i)}}{AUC} = \frac{1}{f_h \times \left[\frac{f_m}{1 + [I]/K_i} \right] + 1 - f_h \times f_m}$$

where K_i is the inhibition constant determined *in vitro*, f_h 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 employed to predict the magnitude of the interaction between LTG and VPA, based on approaches described by Ito *et al.* (1998 and 2004). These were: maximum inhibitor concentration in plasma ($[I]_{max}$), maximum unbound inhibitor concentration in plasma ($[I]_{max.unbound}$), average inhibitor concentration in plasma ($[I]_{ave}$), average unbound inhibitor concentration in plasma ($[I]_{ave.unbound}$), and average and maximum hepatic input concentration given by the inhibitor concentration in the blood plus the inhibitor concentration from GI absorption ($[I]_{inlet}$). The latter parameter was calculated as:

$$[I]_{inlet.max} = f_u \times \left[I_{max} + \frac{k_a \times Dose \times f_a}{Q_H} \right]$$

where $[I]_{max}$ is the maximum inhibitor concentration in blood, k_a is the absorption rate constant, f_a is the fraction of the inhibitor dose absorbed from the GI tract, and Q_H is liver blood flow. The dose was taken as 500mg (3467 μ mol), assuming an 'average' daily dose is 500mg twice daily. It should be noted that increasing the dose to 1000mg resulted in approximately a 9% increase in the AUC ratio (see table 3) determined using the hepatic input concentration. $[I]_{max}$ was taken as the upper limit of the plasma

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VPA therapeutic range for the treatment of epilepsy, that is 100mg/L (693 μ M) (Dutta *et al.* 2003). The lower limit of the VPA therapeutic range is 50mg/L (347 μ M). Thus, the mid-point of the therapeutic range (520 μ M) was used as $[I]_{ave}$. The unbound values of $[I]_{inlet,max}$ and $[I]_{inlet,ave}$ were calculated by multiplying the respective *in vivo* concentrations by the fraction of VPA unbound in blood ($f_{u(VPA)}$), which was taken as 0.1 (Anderson *et al.* 1994). Since 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/hr (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 1000mg/day increased the LTG AUC by 160%. Thus, the *in vivo* LTG AUC ratio ($AUC_{(i)}/AUC$) was taken as 2.60.

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Results

Binding of LTG and VPA to HLM, 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 HLM 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 SD) $f_{u,inc}$ value for LTG binding to BSA was 0.65 ± 0.03 , while for VPA $f_{u,inc}$ ranged from 0.15 ± 0.04 (at $300\mu\text{M}$) to 0.35 ± 0.02 (at $1200\mu\text{M}$). 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 HLM and to BSA and HEK293 cell lysate were 0.49 ± 0.07 and 0.30 ± 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_u values for binding of VPA to mixtures of BSA and HLM and BSA and HEK293 cell lysate were identical to those for binding to BSA alone, ranging from 0.15 ± 0.01 (at $300\mu\text{M}$) to 0.35 ± 0.03 (at $1200\mu\text{M}$). 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 HLM 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 HLM

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Although data were adequately fitted to the two-enzyme Michaelis-Menten equation, LTG N2-glucuronidation by HLM 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 (Figure 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 $2234 \pm 774 \mu\text{M}$ while the mean S_{50} and n values of the Hill component were $1869 \pm 1286 \mu\text{M}$ and 0.65 ± 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 (\pm SD) V_{max} values for the Michaelis-Menten and Hill components were 594 ± 333 and $162 \pm 108 \text{ pmol/min.mg}$, respectively. BSA did not have a significant effect on the mean V_{max} of either component, nor did it affect the Hill coefficient. It should be noted, however, that since the maximum unbound concentration of LTG in incubations conducted in the presence of BSA was approximately $1500 \mu\text{M}$ (ie. maximum total concentration $3000 \mu\text{M}$ 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 HLM in the presence of hecogenin

The highly selective UGT1A4 inhibitor hecogenin (Hec) (Uchaipichat *et al.* 2006b) was added to incubation samples (final concentration $10 \mu\text{M}$) to confirm the involvement of this enzyme in human liver microsomal LTG N2-glucuronidation. Derived kinetic parameters are shown for experiments performed in the presence and absence of BSA (2%) in Table 2. LTG-Gluc formation by HLM in the presence of Hec, with and without 2% BSA, was described by the Hill equation with $n < 1$ (Figure 2), that is Hec

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abolished the Michaelis-Menten component of LTG N2-glucuronidation. In the absence of BSA, the mean (\pm SD) value of S_{50} was $1848 \pm 256\mu\text{M}$. 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_{max} (approximately 30%). The addition of 2% BSA had no effect on the Hill coefficient.

LTG N2-glucuronidation by recombinant UGTs

Activities of UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, 2B15 and 2B17 with 4MU as the substrate were similar to those reported by Uchaipichat *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 (Uchaipichat *et al.* 2006b). Together with UGT2B10 and 2B28, which show very low or no activity towards all substrates tested to date, these enzymes were screened for LTG N2-glucuronidation activity. At an LTG concentration of $1500\mu\text{M}$, UGT1A4 exhibited the highest activity ($105\text{pmol}/\text{min}\cdot\text{mg}$), while 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 (Figure 3). In the absence of BSA, the K_m and V_{max} values for LTG-Gluc formation by UGT1A4 were $1558\mu\text{M}$ and $224\text{pmol}/\text{min}\cdot\text{mg}$, respectively. The addition of 2% BSA resulted in significant ($p<0.05$) increases in both K_m and V_{max} (to $3235\mu\text{M}$ and $1278\text{pmol}/\text{min}\cdot\text{mg}$, respectively). As with previous experiments

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conducted in the presence of BSA, the maximum unbound concentration of LTG in incubations was approximately 1500 μ M, and hence derived kinetic parameters should be considered as estimates. The addition of Hec (10 μ M) to incubations performed with recombinant UGT1A4 decreased the rate of LTG-Gluc formation at substrate concentrations ranging from 10 to 3000 μ M by approximately 95% (data not shown). The low rate of formation of LTG-Gluc by UGT 1A1, 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 (3mM) (Court *et al.* 2003) and the selective UGT2B7 inhibitor fluconazole (2.5mM) (Uchaipichat *et al.* 2006a) inhibited LTG-Gluc formation by pooled HLM at an LTG concentration of 25 μ M by 70% and 74%, respectively, and by 22% and 20%, respectively at a LTG concentration of 1500 μ M (data not shown).

VPA inhibition of LTG-Gluc formation catalyzed by HLM 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 (10mM) added to incubations of pooled HLM essentially abolished the Hill (i.e. non-UGT1A4) component of LTG N2-glucuronidation over the substrate concentration range 25 to 3000 μ M. Kinetic data generated in the presence of VPA (10mM) were well described by the Michaelis-Menten equation (data not shown); derived K_m (1309 μ M) and V_{max} (686pmol/min.mg) were within the ranges obtained for the

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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 μ M) 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 HLM in a competitive manner in the presence and absence of BSA (Figure 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 $387 \pm 12 \mu\text{M}$. 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 co-administered VPA) based on various value of [I] (see 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 to 2.31). However, only calculations that incorporated the total VPA (inhibitor) concentration predicted a clinically significant interaction (predicted AUC ratio 1.86 to 2.31).

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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. Hecogenin (Hec), a highly selective inhibitor of UGT1A4 (Uchaipichat *et al.*, 2006b), 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 HLM in the absence of Hec (viz. $2234 \pm 774 \mu\text{M}$) was similar in value to the K_m for UGT1A4 catalyzed LTG N2-glucuronidation ($1558\mu\text{M}$). 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 ($3.1 - 5.4 \text{ pmol/min.mg}$ versus 105 pmol/min.mg at a substrate concentration of $1500\mu\text{M}$). 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 $25\mu\text{M}$ and $1500\mu\text{M}$, respectively. Zidovudine (a selective substrate for UGT2B7; Court *et al.* 2003) and fluconazole (a selective inhibitor of UGT2B7; Uchaipichat *et al.* 2006a) 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 UGT 1A1, 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

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enzymes are unavailable. It should be noted that the involvement of UGT2B7 in LTG N2-glucuronidation is consistent with recent studies showing 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 ($V_{\max} = 0.79\text{pmol/min.mg}$) 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 ($1000\mu\text{M}$) was low compared to the S_{50} for the putative UGT2B7 component of LTG N2-glucuronidation reported here.

A recent study conducted in this laboratory (Uchaipichat *et al.* 2006a) demonstrated that BSA (2%) reduced the K_m for zidovudine (a selective UGT2B7 substrate) glucuronidation by HLM 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_{\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_{\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 $0.15\mu\text{M}$ (Tsoutsikos *et al.*, 2004). Hence, the addition of exogenous albumin to microsomal incubations may bind inhibitory fatty

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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, PI Mackenzie, JO Miners; manuscript in preparation).

Co-administration 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 (Trapnell *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.* (2006a) 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 ($[I]_{\text{average}}$) to 2.31 ($[I]_{\text{inlet,max}}$). Morris *et al.* (2000) reported that the LTG AUC ratio increased, on average, by 2.6-fold in patients receiving VPA, 1000mg/day (presumed to be 500mg bd). In contrast, use of unbound VPA concentrations in the expression for the AUC ratio did not predict an interaction.

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The non-hyperbolic kinetics for LTG N2-glucuronidation by HLM observed here contrasts to a previous report of Michaelis Menten kinetics (K_m 2560 μ M, V_{max} 0.65nmol/min.mg) for this pathway (Magdalou *et al.* 1992). Substrate concentration ranges differed between the two studies; 12 concentrations in the range 10-3000 μ M in the present study versus five concentrations in the range 500-8000 μ M in the previous work. In order to obtain substrate (LTG) concentrations above 3000 μ 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.* provides non-linear 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 the therapeutic plasma concentration range for LTG is 3 – 14mg/L (12 – 55 μ mol/L), 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 absence, of BSA. Together with the known effect

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of VPA on zidovudine glucuronidation both *in vitro* and *in vivo* (Lertora *et al.* 1994; Trapnell *et al.* 1998; Ethell *et al.* 2003), these data suggest that VPA may inhibit the metabolism of other substrates metabolised by UGT2B7, but not the glucuronidation of predominantly UGT1A4 substrates.

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References

Anderson GD, Gidal BE, Hendryx RJ, Awan AB, Temkin NR, Wilensky AJ, Winn HR (1994). Decreased plasma protein binding of valproate in patients with acute head trauma. *Br J Clin Pharmacol*, 37: 559-662.

Boase S, Miners JO (2002). *In vitro* – *in vivo* correlations for drugs eliminated by glucuronidation: investigation with the model substrate zidovudine. *Br J Clin Pharmacol*, 54: 493-503.

Bowalgaha K, Elliot DJ, Mackenzie PI, Knights KM, Swedmark S, Miners JO (2005). Naproxen and desmethylnaproxen glucuronidation by human liver microsomes and recombinant human UDP-glucuronosyltransferases (UGT): Role of UGT2B7 in the elimination of naproxen. *Br J Clin Pharmacol*, 60: 423-433.

Bressolle F, Gomeni R, Alric R, Royer-Morrot MJ, Necciari J (1994). A double Weibull input function describes the complex absorption of sustained-release oral sodium valproate. *J Pharm Sci*, 83: 1461-4146.

Carlile DJ, Hakooz N, Bayliss MK, Houston JB (1999). Microsomal prediction of *in vivo* clearance of CYP2C9 substrates in humans. *Br J Clin Pharmacol*, 47: 625-635.

Cohen AF, Land GS, Briemer DD, Yuen WC, Winton C, Peck AW (1987). Lamotrigine, a new anticonvulsant: Pharmacokinetics in normal humans. *Clin Pharmacol Ther*, 42: 535-541.

Cotariu D, Zaidman JL (1988). Valproic acid and the liver. *Clin Chem*, 34: 890-977.

DMD#9340

Court MH, Krishnaswamy S, Hoa Q, Duan SX, Patten CJ, von Moltke LL, Greenblatt DJ (2003). Evaluation of 3'-azido-3'-deoxymethymidine, morphine, and codeine as probe substrates for UDP-glucuronosyltransferase 2B7 (UGT2B7) in human liver microsomes: specificity and influence of the UGT2B7*2 polymorphism. *Drug Metab Dispos*, 31: 1125-1133.

Doig MV, Clare RA (1991). Use of thermospray liquid chromatography-mass spectrometry to aid in the identification of urinary metabolites of a novel antiepileptic drug, Lamotrigine. *J Chromatogr*, 554: 181-189.

Dutta S, Cloyd JC, Granneman GR, Collins SD (2003). Oral/intravenous maintenance dosing of valproate following intravenous loading: a simulation. *Epilepsy Res*, 53: 29-38.

Ethell BT, Anderson GD, Burchell B (2003). The effect of valproic acid on drug and steroid glucuronidation by expressed human UDP-glucuronosyltransferases. *Biochem Pharmacol*, 65:1441-9.

Green MD, Tephley TR (1996). Glucuronidation of amines and hydroxylated xenobiotics and endobiotics catalysed by expressed human UGT1.4 protein. *Drug Metab Dispos*, 24: 356-363.

Hiller A, Nguyen N, Strassburg CP, Li Q, Jainta H, Pechstein B, Ruus P, Engel J, Tukey RH, Kronbach T (1999). Retigabine N-glucuronidation and its potential role in enterohepatic circulation. *Drug Metab Dispos*, 27: 605-612.

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Hirsch LJ, Weintraub D, Du Y, Buchsbaum R, Spencer HT, Hager M, Straka T, Bazil CW, Adams DJ, Resor SR, Morrell MJ (2004). Correlating lamotrigine serum concentrations with tolerability in patients with epilepsy. *Neurology*, 63: 1022-1026.

Ito K, Iwatsubo T, Kanamitsu S, Ueda K, Suzuki H, Sugiyama Y (1998). Prediction of pharmacokinetic alterations caused by drug-drug interactions: Metabolic interactions in the liver. *Pharmacol Rev*, 50: 387-411.

Ito K, Brown HS, Houston BJ (2004). Database analysis for the prediction of *in vivo* drug-drug interactions from *in vitro* data. *Br J Clin Pharmacol*, 57: 473-486.

Jin CJ, Miners JO, Lillywhite KJ, Mackenzie PI (1993). Complementary deoxyribonucleic acid cloning and expression of a human liver uridine diphosphate-glucuronosyltransferase glucuronidating carboxylic acid containing drugs. *J Pharmacol Exp Ther*, 264 : 475-479.

Jin CJ, Mackenzie PI, Miners JO (1997). The regio- and stereo- selectivity of C19 and C21 hydroxysteroid glucuronidation by UGT2B7 and UGT2B11. *Arch Biochem Biophys* 341: 207-211.

Kiang TK, Ensom MH, Chang TK (2005). UDP-Glucuronosyltransferases and clinical drug-drug interactions. *Pharmacol Ther*, 106: 97-132.

Lertora JLL, Rege AB, Greenspan DL, Akula S, George WJ, Hyslop NE, Agrawal KC (1994). Pharmacokinetic interaction between zidovudine and valproic acid in patients infected with human immunodeficiency virus. *Clin Pharmacol Ther*, 56: 272-278.

DMD#9340

Ludden LK, Ludden TM, Collins JM, Pentikis HS, Strong JM (1997). Effect of albumin on estimations, *in vitro*, of phenytoin Vmax and Km values; implications for clinical correlation. *J Pharmacol Exp Ther*, 282: 391-396.

Mackenzie PI, Bock KW, Burchell B, Giullemette C, Ikushiro S, Iyanagi T, Miners JO, Owens IS, Nebert DW (2005). Nomenclature update for the mammalian UDP-glucuronosyltransferase (UGT) gene superfamily. *Pharmacogenet Genomics*, 75: 677-685.

Magdalou J, Herber R, Bidault R, Seist G (1992). *In vitro* predictions of a novel antiepileptic drug, lamotrigine, by human liver microsomes. *J Pharmacol Exp Ther*, 260: 1166-1173.

McLure JA, Miners JO, Birkett DJ (2000). Non-specific binding of drugs to human liver microsomes. *Br J Clin Pharmacol*, 49: 453-461.

Mikati MA, Schachter SC, Schomer DL, Keally M, Osborne-Shafer P, Seaman CA, Sheridan PH, Ashworth M, Kupferberg H, Valakas A (1989). Long-term tolerability, pharmacokinetic and preliminary efficacy study of lamotrigine in patients with resistant partial seizures. *Clin Neuropharmacol*, 12: 312-321.

Miners JO, Lillywhite KJ, Matthews AP, Jones ME, Birkett DJ (1988). Kinetic and inhibitor studies of 4-methylumbelliferone and 1-naphthol glucuronidation by human liver microsomes. *Biochem Pharmacol*, 37: 665-671.

Miners JO, Smith PA, Sorich MJ, McKinnon RA, Mackenzie PI (2004). Predicting human drug glucuronidation parameters: application of *in vitro* and *in silico* modeling approaches. *Annu Rev Pharmacol Toxicol*, 44: 1-25.

DMD#9340

Morris RG, Black AB, Lam E, Westley IS (2000). Clinical study of lamotrigine and valproic acid in patients with epilepsy: using a drug interaction to advantage? *Ther Drug Monit*, 22: 656-660.

Ohta Y, Fukushima S, Yamashita N, Niimi T, Kubota T, Akizawa E, Koizumi O (2005). UDP-Glucuronosyltransferase 1A1 directly binds to albumin. *Hepatol Res* 31: 241-245.

Pellock JM (1994). The clinical efficacy of lamotrigine as an antiepileptic drug. *Neurology*, 44: S29-S35.

Sinz MW, Rimmel RP (1991). Analysis of lamotrigine and lamotrigine 2-N-glucuronide in guinea pig blood and urine by reverse-phase ion-pairing liquid chromatography. *J Chromatogr*, 571: 217-230.

Soars MG, Burchell B, Riley RJ (2002). *In vitro* analysis of human drug glucuronidation and prediction of *in vivo* metabolic clearance. *J Pharmacol Exp Ther*, 301:382-390.

Sorich MJ, Smith PA, McKinnon RA, Miners JO (2002). Pharmacophore and quantitative structure activity relationship modelling of UDP-glucuronosyltransferase 1A1 (UGT1A1) substrates. *Pharmacogenetics*, 12: 635-645.

Staines AG, Coughtrie MJH, Burchell B (2004). N-glucuronidation of carbamazepine in human tissue is mediated by UGT2B7. *J Pharmacol Exp Ther*, 311: 1131-1137

DMD#9340

Stone AN, Mackenzie PI, Galetin A, Houston JB, Miners JO (2003). Isoform selectivity and kinetics of morphine 3- and 6-glucuronidation by human UDP-Glucuronosyltransferases: evidence for atypical glucuronidation kinetics by UGT2B7. *Drug Metab Dispos*, 31: 1086-1089.

Tang C, Lin Y, Rodrigues AD, Lin JH (2002). Effect of albumin on phenytoin and tolbutamide metabolism in human liver microsomes: an impact more than protein binding. *Drug Metab Dispos*, 30: 648-654.

Trapnell CB, Klecker RW, Jamis-Dow C, Collins JM (1998). Glucuronidation of 3'-azido-3'-deoxythymidine (zidovudine) by human liver microsomes: relevance to clinical pharmacokinetic interactions with atovaquone, fluconazole, methadone and valproic acid. *Antimicrob Agents Chemother*, 42: 1592-1596.

Tsoutsikos P, Miners JO, Stapleton A, Thomas A, Sallustio BC, Knights KM (2004). Evidence that unsaturated fatty acids are potent inhibitors of renal UDP0glucuronosyltransferases (UGT): kinetic studies using human kidney cortical microsomes and recombinant UGT1A9 and UGT2B7. *Biochem Pharmacol* 67: 191-199.

Uchaipichat V, Mackenzie PI, Guo XH, Gardner-Stephen D, Galetin A, Houston JB, Miners JO (2004). Human UDP-Glucuronosyltransferases: isoform selectivity and kinetics of 4-methylumbelliferone and 1-naphthol glucuronidation, effects of organic solvents, and inhibition by diclofenac and probenecid. *Drug Metab Dispos*, 32: 413-423.

Uchaipichat V, Winner LK, Mackenzie PI, Elliot DJ, Williams JA, Miners JO (2006a). Quantitative prediction of *in vivo* inhibitory drug interactions involving glucuronidated

DMD#9340

drugs from *in vitro* data: The effect of fluconazole on zidovudine glucuronidation. *Br J Clin Pharmacol.* 61 : 427-439.

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

Vinik A (2005). Clinical review: Use of antiepileptic drugs in the treatment of chronic painful diabetic neuropathy. *J Clin Endocrinol Metab*, 90: 4936-4945.

Zhang D, Zhao W, Roongta VA, Mitroka JG, Klunk LJ, Zhu M (2004). Amide N-glucuronidation of MaxiPost catalyzed by UDP-glucuronosyltransferase 2B7 in humans. *Drug Metab Dispos*, 32: 545-551.

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Footnote

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Legends for Figures

Figure 1 – Structures of lamotrigine and lamotrigine N2-glucuronide.

Figure 2 – Representative velocity versus substrate concentration and Eadie Hofstee plots for LTG N2-glucuronidation by human liver microsomes (HLM), with and without BSA (2%). Plots are shown for incubations conducted in the presence and absence of hecogenin (Hec). Units of $V/[S]$ are pmol glucuronide/ $\mu\text{M}\cdot\text{min}\cdot\text{mg}$. Points are experimentally determined values, while lines are from model – fitting.

Figure 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/ $\mu\text{M}\cdot\text{min}\cdot\text{mg}$. Points are experimentally determined values, while lines are from model – fitting.

Figure 4 – Representative Dixon plots for the inhibition of LTG N2-glucuronidation by VPA in the presence of hecogenin (10 μM). Plots are shown for incubations conducted in the presence and absence of BSA (2%). Points are experimentally determined values, while lines are from model – fitting.

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Table 1 – Mean derived kinetic parameters for LTG N2-glucuronidation by human liver microsomes, with and without BSA

		Hill component			Michaelis-Menten component	
		S ₅₀ (μ M)	V _{max1} (pmol/min.mg)	n	K _m (μ M)	V _{max2} (pmol/min.mg)
Without BSA	Mean	1869	162	0.65	2234	594
	SD	1286	108	0.16	774	333
	Range	748 - 3999	82 - 346	0.56 - 0.87	1487 - 3435	347 - 1158
With BSA	Mean	255	99	0.59	2342	694
	SD	170	46	0.16	1118	299
	Range	102 - 504	63 - 177	0.41 - 0.80	1217 - 3614	289 - 1085

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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

		S₅₀ (μM)	V_{max} (pmol/min.mg)	n
Without BSA	Mean	1848	235	0.74
	S.D	256	29	0.04
	Range	783 - 2283	155 - 286	0.69 - 0.87
With BSA	Mean	382	156	0.71
	S.D	81	18	0.01
	Range	223 - 690	106 - 212	0.68 - 0.74

Table 3 – Predicted mean in vivo LTG AUC ratios calculated from K_i values generated in the absence and presence of BSA

		<i>In vitro</i> K_i (μM)	Predicted AUC ratio based on:							
		$[I]_{\text{max}}$	$[I]_{\text{max.unbound}}$	$[I]_{\text{ave}}$	$[I]_{\text{ave.unbound}}$	$[I]_{\text{inlet.max}}$	$[I]_{\text{inlet.max.unbound}}$	$[I]_{\text{inlet.ave}}$	$[I]_{\text{inlet.ave.unbound}}$	
Without BSA	Mean	2465	1.22	1.02	1.17	1.00	1.28	1.03	1.23	1.02
	SD	370	0.03	0.01	0.03	0.00	0.04	0.00	0.03	0.00
	Range	1988 - 2987	1.18 - 1.26	1.01 - 1.02	1.14 - 1.20	1.00 - 1.00	1.23 - 1.34	1.02 - 1.04	1.19 - 1.28	1.02 - 1.03
With BSA	Mean	387	2.08	1.10	1.86	1.01	2.31	1.18	2.12	1.15
	SD	12	0.05	0.01	0.04	0.00	0.05	0.01	0.05	0.01
	Range	361 - 430	1.99 - 2.13	1.09 - 1.11	1.79 - 1.91	1.01 - 1.01	2.21 - 2.37	1.16 - 1.19	2.04 - 2.18	1.13 - 1.16

Figure 1

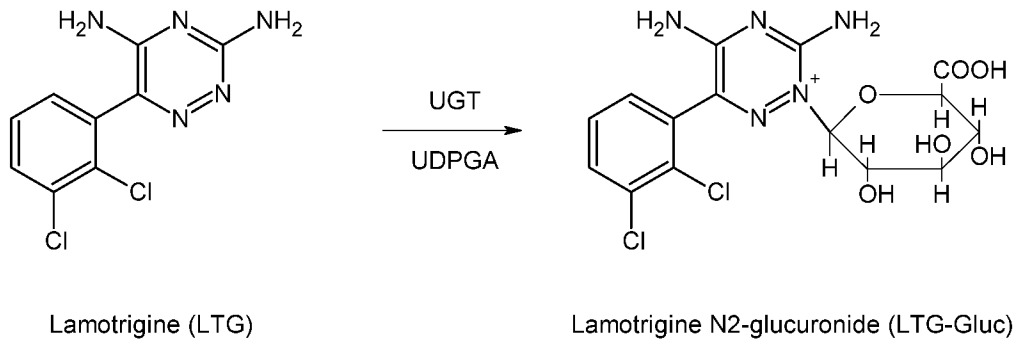


Figure 2

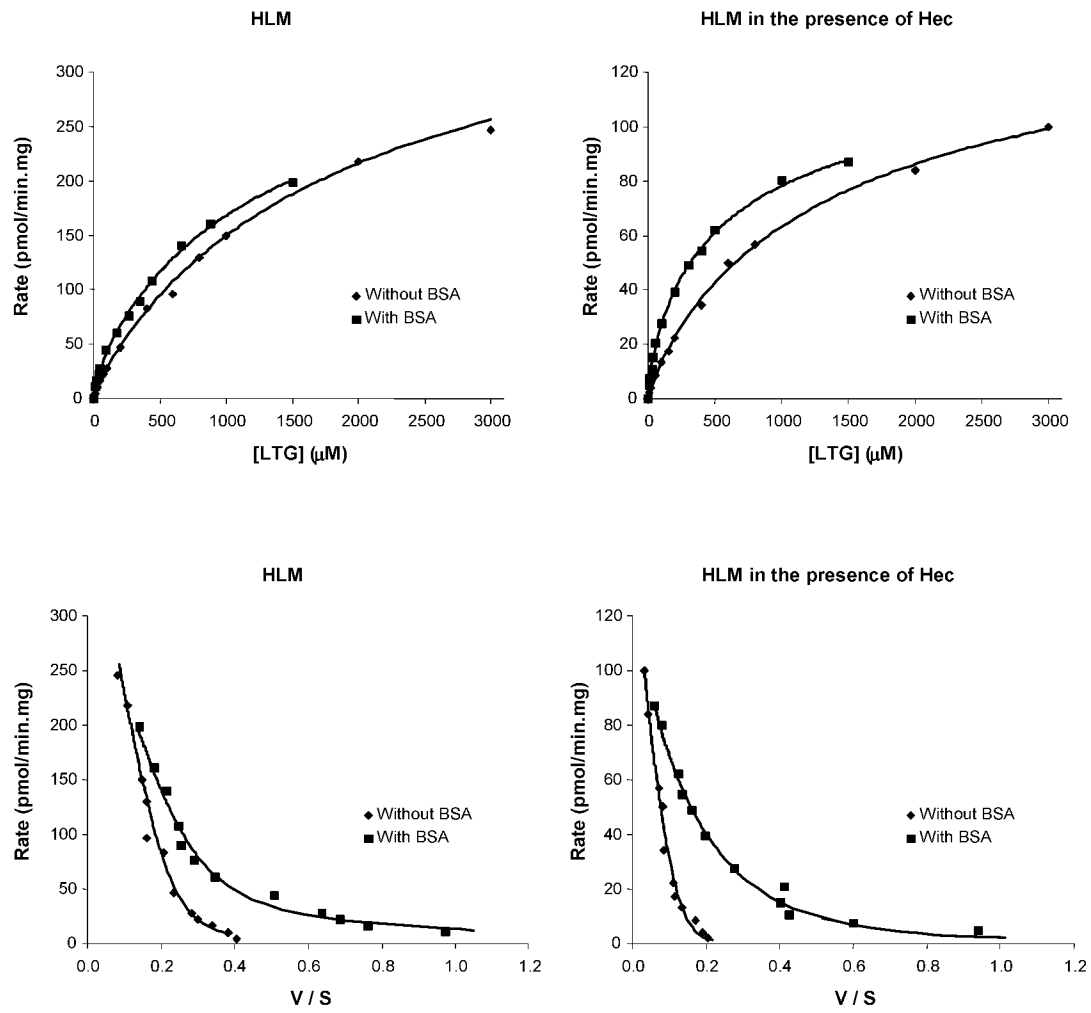


Figure 3

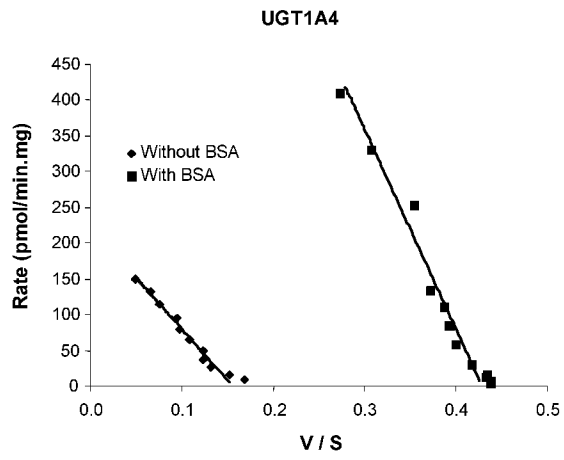


Figure 4

