Transporter-mediated uptake of UDP-glucuronic acid by human liver microsomes: Assay conditions, kinetics and inhibition.

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Running Title: Kinetics and inhibition of UDP-GlcUA uptake by HLM

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Abbreviations:
4-methylumbelliferone; 4MU, human liver microsomes; HLM, UDP-glucuronic acid; UDP-GlcUA, UDP-N-acetylglucosamine; UDP-GlcNAc, UDP-glucuronosyltransferases; UGT, ultra-high performance liquid chromatography - mass spectrometry; UPLC-MS.
Abstract

This study characterized the kinetics, variability and factors that affect UDP-glucuronic acid (UDP-GlcUA) uptake by human liver microsomes (HLM). Biphasic kinetics were observed for UDP-GlcUA uptake by HLM. Uptake affinities (assessed as $K_d$) of the high- and low-affinity components differed by more than an order of magnitude (13±6µM versus 374±175µM), but were comparable in terms of the maximal rate of uptake with mean $V_{max}$ values differing less than 2.3-fold (56±26pmol/min/mg versus 131±35pmol/min/mg). Variability in total intrinsic transporter activity ($U_{int}$) for microsomal UDP-GlcUA uptake across 12 livers was less than 4-fold. Experiments performed to optimize the conditions for microsomal UDP-GlcUA uptake demonstrated that both components were trans-stimulated by pre-loading (luminal addition) with an alternate UDP-sugar, and essentially abolished by the thiol-alkylating agent N-ethylmaleimide. Furthermore, interaction studies undertaken with a panel of drugs, alternate UDP-sugars and glucuronide conjugates, at low- (2.5µM) and high- (1000µM) UDP-GlcUA concentrations, demonstrated that both components were inhibited to varying extents. Notably, the nucleoside analogs zidovudine, stavudine, lamivudine and acyclovir inhibited both the high- and low- affinity components of microsomal UDP-GlcUA uptake by >45% at an inhibitor concentration of 100µM. Taken together, these data demonstrate that human liver microsomal UDP-GlcUA uptake involves multiple protein-mediated components, and raises the possibility of impaired in vivo glucuronidation activity resulting from inhibition of UDP-GlcUA uptake into the ER membrane by drugs and other compounds.
Introduction

Glucuronidation serves as a key metabolic pathway for numerous endogenous compounds including bilirubin, bile acids, fatty acids, and steroid and thyroid hormones (Burchell et al. 1995, Radominska-Pandya et al. 1999, Tukey and Strassburg 2000, Miners et al. 2004, Kiang et al. 2005, Knights et al. 2013) and is an essential clearance mechanism for drugs from many therapeutic classes, including but not limited to: analgesics, non-steroidal anti-inflammatory agents, anticonvulsants, antipsychotics, antivirals and benzodiazepines (Miners and Mackenzie 1991, Kiang et al. 2005, Sorich et al. 2006, Miners et al. 2010). The glucuronidation reaction, which is catalyzed by the UDP-glucuronosyltransferases (UGT) superfamily of enzymes, involves the covalent linkage (conjugation) of glucuronic acid, derived from the cofactor UDP-glucuronic acid (UDP-GlcUA), to a substrate bearing a suitable functional group (Miners and Mackenzie 1991, Radominska-Pandya et al. 1999, Tukey and Strassburg 2000).

As the catalytic domain of UGT is located on the luminal side of the endoplasmic reticulum (ER) membrane (Radominska-Pandya et al. 1999, Miners et al. 2004, Rowland et al. 2013) and UDP-GlcUA is synthesized in the cytosol (Radominska-Pandya et al. 1999), uptake of UDP-GlcUA into the luminal compartment of the ER is an essential step for glucuronidation. However, UDP-GlcUA is a highly polar molecule that is unable to diffuse passively across the ER membrane. While the exact mechanism of luminal UDP-GlcUA uptake remains unclear (Meech and Mackenzie 1997, Radominska-Pandya et al. 1999, Kobayashi et al. 2006), it is generally accepted that this process is mediated by specific protein transporter(s).

In this regard, it has been demonstrated that carrier mediated transport of UDP-GlcUA across the ER membrane is necessary for glucuronidation in rat liver microsomal vesicles and permeabilized hepatocytes (Bossuyt and Blankaert 1997). The role of protein transporter(s) in
microsomal UDP-GlcUA uptake is supported by data demonstrating that this process may be inhibited by the anion transport inhibitor 4-acetamido-4’-isothiocyanostilbene-2,2’-disulfonic acid and by the thiol-alkylating agent N-ethylmaleimide (Berg et al. 1995, Bossuyt and Blankaert 1994). It has additionally been shown that the presence of UDP-N-acetylglucosamine (UDP-GlcNAc) in the lumen of rat liver microsomes causes trans-stimulation of rat liver microsomal UDP-GlcUA uptake (Bossuyt and Blankaert 1995). Kinetic studies with rat liver microsome vesicles indicate the existence of two distinct components to UDP-GlcUA uptake, which can be differentiated on the basis of UDP-GlcUA binding affinity (Battaglia et al. 1996, Kobayashi et al. 2006). By contrast, there have been no studies that have investigated the transport of UDP-GlcUA by human liver microsomes or the factors that influence UDP-GlcUA transport in human liver.

The approaches used to quantify UDP-GlcUA uptake have typically employed sub-optimal assay conditions and a non-selective radioisotopic filtration technique. Accordingly, previous studies have produced conflicting results regarding luminal UDP-GlcUA uptake (Battaglia et al 1996, Muraoka et al. 2001, Kobayashi et al. 2006). Thus, this study sought to develop and validate a sensitive and specific ultra-high performance liquid chromatography - mass spectrometry (UPLC-MS) based approach to characterize the uptake of UDP-GlcUA by human liver microsomes (HLM). Specifically, experiments where undertaken to: (i) establish an assay and optimize conditions for the reliable measurement of UDP-GlcUA uptake by HLM, (ii) characterize the kinetics of UDP-GlcUA uptake by HLM from individual donors and assess the inter-individual variability in this process, and (iii) examine the capacity of a panel of drugs, alternate UDP-sugars and glucuronide conjugates to inhibit UDP-GlcUA uptake. It was demonstrated that, as for non-human tissues (Battaglia et al 1996, Kobayashi et al. 2006), there are two kinetically distinct components for UDP-GlcUA uptake, both of which are transporter-mediated. Furthermore, it shown that both components may be stimulated by pre-incubation of HLM with alternate UDP sugars, or inhibited to a varying
extent by co-incubation in the presence of alternate UDP-sugars, drugs and glucuronide conjugates.
**Materials and Methods**

**Materials**

4-methylumbelliferone glucuronide, acyclovir, frusemide, β-estradiol 3-glucuronide, β-estradiol 17-glucuronide, lamivudine, N-ethylmaleimide, probenecid, stavudine, UDP-glucose (UDP-Glc), UDP-glucuronic acid (UDP-GlcUA), UPD-N-acetyl-glucosamine (UDP-GlcNAc), zidovudine and zidovudine glucuronide were obtained from Sigma-Aldrich (Sydney, AUS). Acetaminophen glucuronide, 1-hydroxypyrene glucuronide, morphine 3-glucuronide, phenolphthalein glucuronide, propofol glucuronide and SN-38 glucuronide were purchased from Toronto Research Chemicals (Ontario, Canada). MF-Millipore mixed cellulose esters membrane filters (pore sizes 0.025, 0.22 and 0.45µm; diameter 25mm) were obtained from Millipore (Sydney, AUS). All other materials used were analytical reagent grade or of the highest grade available.

**Preparation of human liver microsomes**

Human liver microsomes (HLM) were freshly prepared from 12 individual livers (H7, H10, H11, H12, H13, H23, H24, H25, H27, H29, H30 and H40), obtained from the human liver ‘bank’ of the Department of Clinical Pharmacology, 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. HLM were prepared by differential centrifugation, as described by Bowalgaha et al. (2005). The final microsomal pellets were suspended in 0.1M phosphate buffer (pH 7.4) and aliquots stored at −80 °C. Microsomes were invariably used within 14 days of preparation. The protein concentration of microsomal preparations was determined according to the method of Lowry et al. (1951).

**Kinetics of microsomal UDP-GlcUA uptake**

Prior to uptake experiments, HLM were conditioned by incubating at 37°C for 90min in transport buffer (phosphate buffer (20mM; pH 7.4) containing sucrose (250mM), potassium chloride (150mM) and magnesium chloride (5mM)), then preloaded with UDP-GlcNAc by
incubating for a further 90min at 37°C in the presence of UDP-GlcNAc (2mM). The conditioned HLM pellet was separated by centrifugation (30000g) and resuspended in transport buffer. The kinetics of microsomal uptake of UDP-GlcUA were characterized using a modification of the procedure of Kobayashi et al. (2006). Samples (200µL) contained conditioned HLM (5mg/mL) and NAD+ (2mM) in transport buffer. Incubations were initiated by the addition of UDP-GlcUA (5-1000µM) and continued for 10min at 37°C in a shaking water-bath. Following incubation, samples were immediately filtered through a pre-soaked (transport buffer) MF-membrane filter (0.025µm pore size; Millipore, Sydney, AUS) attached to a vacuum manifold (Type 1225; Millipore, Sydney, AUS). The membrane was washed with 5mL of ice-cold transport buffer and suspended in ice-cold 50:50 methanol/water (containing hydrochloric acid; 0.1% v/v), vortex mixed and centrifuged (6000g). A 5µL aliquot of the supernatant fraction was analyzed by UPLC-MS. The effects of microsomal conditioning, freeze-thawing of HLM, and the addition of alamethacin, NAD+ and N-ethylmaleimide to incubations were investigated. For experiments performed in the presence of alamethacin, HLM were conditioned by preloading with UDP-GlcNAc (2mM) as described above, then incubated with alamethacin (50µg/mg protein) for 20min prior to the addition of UDP-GlcUA. Control experiments, whereby HLM were incubated for 20min in the absence of alamethacin prior to the addition of UDP-GlcUA, demonstrated that in the absence of alamethacin, the additional incubation step did not affect UDP-GlcUA uptake. The rate of UDP-GlcUA uptake by HLM was linear with respect to incubation time to 15min and HLM concentration to 10mg/mL.

**Inhibition of microsomal UDP-GlcUA uptake**

The capacity of alternate UDP-sugars (UDP-Glc and UDP-GlcNAc; 100µM), nucleoside analogs (acyclovir, lamivudine, stavudine and zidovudine; 100µM), anion transporter inhibitors (frusemide and probenecid; 2000µM), and representative glucuronide conjugates (4-methylumbelliferone glucuronide, acetaminophen glucuronide, β-estradiol 3-glucuronide, β-estradiol 17-glucuronide, 1-hydroxypyrene glucuronide, morphine 3-glucuronide,
phenolphthalein glucuronide, propofol glucuronide, SN-38 glucuronide and zidovudine glucuronide; 100µM) to inhibit microsomal UDP-GlcUA uptake was evaluated by co-incubation with these compounds. Potential inhibitors were added at to incubations at the same time as UDP-GlcUA, thereby representing cytosolic (cis)- addition. Microsomal uptake of UDP-GlcUA in the presence of the potential inhibitors was assessed at low (2.5µM) and high (1000µM) UDP-GlcUA concentrations to elucidate the effects of potential inhibitors on the high- and low- affinity components of microsomal UDP-GlcUA uptake, respectively. Incubation conditions were as described previously for the assessment of the kinetics of microsomal UDP-GlcUA uptake. Control experiments were performed to assess the effects of the solvents dimethyl sulfoxide (1% v/v) and methanol (1% v/v) (which were used to dissolve potential inhibitors) on microsomal UDP-GlcUA uptake. Consistent with the findings of Battaglia et al. (1996), neither solvent affected microsomal UDP-GlcUA uptake to a measurable extent. Microsomal UDP-GlcUA uptake was also assessed in the presence of the protein modifying agent N-ethylmaleimide (2000µM).

Non-specific binding

To exclude confounding non-specific binding of UDP-GlcUA to HLM, binding of UDP-GlcUA to a pool (n=12) of HLM was measured by equilibrium dialysis according to the method of McLure et al. (2000). Binding measurements were performed using a Dianorm equilibrium dialysis apparatus that comprised Teflon dialysis cells (capacity of 1.2 mL/side) separated into two compartments with Sigma-Aldrich dialysis membrane (molecular mass cut off, 12kDa). One side of the dialysis cell was loaded with 1mL of UDP-GlcUA (5-1000µM) in phosphate buffer (0.1M, pH7.4). The other compartment was loaded with 1mL of HLM (5mg/mL) in phosphate buffer (0.1M, pH7.4). The dialysis cell assembly was immersed in a water bath maintained at 37°C and rotated at 12rpm for 4hr. Control experiments were performed with phosphate buffer or HLM on both sides of the dialysis cell at low and high concentrations of UDP-GlcUA to ensure that equilibrium was attained. A 200µL aliquot was collected from each compartment, treated with ice-cold methanol containing hydrochloric...
acid (0.1%; 200µL), and cooled on ice. Samples were subsequently centrifuged at 4000g for 10min at 10°C, and an aliquot of the supernatant fraction (5µL) was analyzed by UPLC-MS. The binding of UDP-GlcUA to HLM was calculated as the concentration of UDP-GlcUA in the buffer compartment divided by the concentration of UDP-GlcUA in the HLM.

**Quantification of UDP-GlcUA**

Chromatography was performed using a Waters Acquity™ UPLC system fitted with a Waters Acquity™ UPLC HSS T3 C18 (2.1 x 150mm, 1.8μm particle size) analytical column (Waters Corporation, Sydney, AUS). UDP-GlcUA was separated from matrix components using a gradient mobile phase comprising 10mM ammonium acetate (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.2mL/min. The elution gradient was increased linearly from 2.5% B to 90% B over 3min. The total run time, including reconditioning of the column to initial conditions, was 5min. The retention time for UDP-GlcUA was 1.49min. Column elutant was monitored by mass spectrometry, performed on a Waters Q-ToF Premier™ quadrupole, orthogonal acceleration time-of-flight tandem mass spectrometer (Waters Corporation, Sydney, AUS) operated in negative ion mode with electrospray ionization (ESI) (Figure 1). The nebulizer and cone gases were set to flow rates of 400L/hr and 50L/hr, respectively. Desolvation and source temperature were set at 225°C and 90°C, respectively. The capillary and cone voltages were set to 2400V and 40V, respectively. Time-of-flight (ToF) data were collected in selected ion (MS²) mode, with the precursor m/z set to 578. Data were collected between m/z 400 and 700 at collision energy of 3eV. Resulting mass spectra were analyzed using Waters QuanLynx™ software (Waters Corporation, Sydney, Australia). UDP-GlcUA was detected at an m/z of 578.0186 [M-H] (Figure 1). The lower limit of quantification for UDP-GlcUA, defined as 10 times the background noise, was 60nM (0.1μg/L). The linear detection range for UDP-GlcUA was 0.06 to 100µM. This range spanned from 8-fold lower than the minimum concentration observed in the retained fraction of filtered incubation samples (0.5µM) to 5-fold higher than the maximum concentration observed in the retained fraction of filtered incubation samples (20µM). Within-day assay...
reproducibility was assessed in 8 separate incubations of the same batch of ‘pooled’ HLM. Coefficients of variation were 2.7% and 1.5% at UDP-GlcUA concentrations of 2.5µM and 1000µM, respectively.

4-Methylumbelliferone glucuronidation activity and detection of glucuronide conjugates

The capacity of filtered and unfiltered HLM samples to catalyze the glucuronidation of 4-methylumbelliferone (4MU) was assessed in order to characterize the glucuronidation activity of filtered HLM protein and to exclude utilization of UDP-GlcUA via aglycone glucuronidation as a confounding factor during uptake experiments performed in the presence of potential inhibitors, using the incubation conditions of Rowland et al. (2008).

Chromatography was performed using a Waters Acquity™ UPLC system fitted with a Waters Acquity™ UPLC HSS T3 C18 (2.1 x 150mm, 1.8μm particle size) analytical column (Waters Corporation, Sydney, AUS). Glucuronide conjugates were separated from matrix components using a gradient mobile phase comprising 10mM ammonium formate (adjusted to pH3.0 with formic acid; mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.25mL/min. The elution gradient was increased linearly from 5% B to 75% B over 5min. The total run time, including reconditioning of the column to initial conditions, was 7min. Column eluant was monitored by mass spectrometry, performed on a Waters Q-ToF Premier™ quadrupole, orthogonal acceleration time-of-flight tandem mass spectrometer (as described for quantification of UDP-GlcUA uptake) operated in negative ion mode. The nebulizer and cone gases were set to flow rates of 400L/hr and 50L/hr, respectively. Desolvation and source temperature were set at 280°C and 90°C, respectively. The capillary and cone voltages were set to 2600V and 50V, respectively. Time-of-flight (ToF) data were collected in MS mode, with data were collected between m/z 100 and 1000Da at collision energy of 5eV.
Data analysis

UDP-GlcUA uptake kinetic data are presented as the mean of duplicate experiments (<10% variance) for individually prepared HLM. $K_d$ values were determined by fitting a two component transporter equation to experimental data:

$$V = \frac{V_{\text{max}1} \times [S]}{K_{d1} + [S]} + \frac{V_{\text{max}2} \times [S]}{K_{d2} + [S]}$$

(1)

where $V$ is the rate of uptake, $V_{\text{max}n}$ is the maximum rate of uptake attributed to the $n^{th}$ transporter mode, $K_{dn}$ is the dissociation constant for the $n^{th}$ transporter mode, and $[S]$ is the total substrate concentration. Intrinsic transporter activity ($U_{\text{int}}$) was calculated as $V_{\text{max}}/K_d$.

Fitting was performed using EnzFitter (version 2.0.18.0; Biosoft, Cambridge, UK) based on the unbound UDP-GlcUA concentration present in incubations. In all cases, goodness-of-fit parameters ($R^2$ values, F statistic, standard error of the fit parameter) for data described by the two component transporter equation were superior to the parameters generated from fitting with the single component transporter equation or Hill equation (i.e. negative co-operativity). The superior fit of the two component transporter equation was confirmed by visual inspection of the fitted data. Inhibition data presented in Figure 3 are the mean (±SD) of quadruplicate experiments for pooled HLM.
Results

Assay for UDP-GlcUA transport by HLM

The capacity of mixed cellulose ester membrane filters of varying pore size to retain HLM (5mg/mL) suspended in transport buffer was assessed by measurement of the protein concentration of filtered and unfiltered samples. Membranes of 0.025, 0.22 and 0.45µm pore size retained 96, 61 and 44% of added protein, respectively. The presence and identity of filtered proteins was further investigated by comparison of 4-MU glucuronidation activity in filtered and unfiltered samples. When normalized for protein concentration, samples filtered through 0.22 and 0.45µm membranes exhibited comparable 4-MU glucuronidation activity to unfiltered samples; K_m and V_max values from unfiltered, 0.22µm filtered and 0.45µm filtered samples were 80µM and 69500pmol/min/mg, 78µM and 71700pmol/min/mg, and 82µM and 68300pmol/min/mg, respectively. Negligible 4-MU glucuronidation activity was observed in the samples filtered through a 0.025µm membrane.

Optimization of UDP-GlcUA uptake assay conditions

Incubation conditions for microsomal UDP-GlcUA uptake were optimized for transporter activity. Experiments were additionally performed to exclude confounding factors such as non-specific binding of UDP-GlcUA to matrix components and to assess the contribution of protein mediated pathway(s). In this regard, UDP-GlcUA was not detected in the retentate of filtered incubation samples containing the pore forming peptide alamethacin, demonstrating that washing with 5mL of ice-cold transport buffer was sufficient to remove all non-internalized UDP-GlcUA from the reservoir compartment in the presence of incubation samples. Equilibrium dialysis experiments confirmed that non-specific binding of UDP-GlcUA to ‘pooled’ HLM (5mg/mL) was negligible (<15%) across the UDP-GlcUA concentration range 5 µM to 1000µM. Similarly, no uptake of UDP-GlcUA was observed when the protein modifying agent N-ethylmaleimide (2000 µM) was added to samples.
Consistent with studies undertaken with rat hepatic microsomes and expressed cell lines (Bossuyt and Blanckaert 1995 and 1997, Muraoka et al. 2001), UDP-GlcUA uptake was reduced by 63±2% when HLM were not pre-loaded with UDP-GlcNAc. This effect was independent of UDP-GlcUA concentration across the range 50µM to 1000µM. Similarly, consistent with previous reports describing the requirement for freshly prepared rat microsomes (Bossuyt and Blanckaert 1994a; Battaglia et al. 1996), repeated freezing and thawing of ‘pooled’ HLM (0 to 4 cycles) caused a reduction in microsomal UDP-GlcUA uptake due to decreases in both affinity (increased $K_d$) and capacity (decreased $V_{max}$). By way of example, following four freeze thaw cycles, $K_d$ values for the high- and low- affinity components of UDP-GlcUA uptake by pooled HLM increased from 12 to 17µM and from 348 to 487µM, respectively. The magnitude of the effect was proportional to the number of freeze-thaw cycle; a 40% reduction in total $U_{int}$ for UDP-GlcUA uptake was observed following two ‘freeze-thaw’ cycles (4.9 versus 6.9µL/min/mg), while a 70% reduction in total $U_{int}$ was observed following four ‘freeze-thaw’ cycles (4.9 versus 8.3µL/min/mg).

A decrease in UDP-GlcUA concentration was also observed in unfiltered samples that were incubated at 37°C for 10min in the absence of NAD+. This decrease was concentration dependent, ranging from 80% to 25% at UDP-GlcUA concentrations of 50µM and 1000µM, respectively. No decrease in UDP-GlcUA concentration was observed for unfiltered incubation samples in the presence of NAD+ (2mM). As such 2mM NAD+ was routinely included in all incubation samples. This observation is consistent with a previous report that, in detergent disrupted HLM, the presence of NAD+ impaired the glucuronidation of 4-MU (a pan-UGT substrate) either through direct inhibition of the UGT enzyme or some alternate mechanism, with an IC$_{50}$ value of approximately 350µM (Ishii et al. 2012). The presence of NAD+ (2mM) in incubation samples did not affect the kinetics of microsomal UDP-GlcUA uptake.
**Kinetics of microsomal UDP-GlcUA uptake**

Kinetic data for microsomal UDP-GlcUA uptake were well described by the equation for the two component transporter model (Figure 2). $K_d$ values for the high- and low- affinity components in individual human livers are shown in Table 1. The mean (± S.D) $K_d$ values for the high- and low- affinity components were 13±5.9µM and 374±175µM, respectively. Notably, inter- liver variability in the affinity for both components was relatively minor (< 5-fold), with $K_d$ values ranging from 5.1 to 23µM and 139 to 610µM for the high- and low-affinity components, respectively. Mean (± S.D) $V_{max}$ values for the high- and low- affinity components were 56 ±26 pmol/min/mg and 131±35 pmol/min/mg, respectively. $U_{int}$ was an order of magnitude higher for the high affinity component of microsomal UDP-GlcUA uptake. As with binding affinity, inter- liver variability in capacity of the two components was minor (< 5-fold), with $V_{max}$ values ranging from 18 to 92 pmol/min/mg and 86 to 196 pmol/min/mg for the high- and low- affinity components, respectively.

**Inhibition of microsomal UDP-GlcUA uptake**

The ability of alternate UDP-sugars, anion transport inhibitors (reported inhibitors of SLC35 transporter proteins), nucleotide reverse transcriptase inhibitors and glucuronide conjugates (100µM) to inhibit UDP-GlcUA uptake was assessed at a low (2.5µM) and high (1000µM) UDP-GlcUA concentrations (Figure 3). Substitution of the mean kinetic parameters given in Table 1 into equation 1 (Data Analysis) indicates that the high affinity component is responsible for 90% of uptake at a UDP-GlcUA concentration of 2.5 µM, while the low affinity component is responsible for ~70% of uptake at a UDP-GlcUA concentration of 1000µM. UDP-Glc and UDP-GlcNAc inhibited the high affinity component of microsomal UDP-GlcUA uptake by 70% and 80%, respectively, and the low affinity component of microsomal UDP-GlcUA uptake by 40% and 55%, respectively. When added at a concentration of 100µM, the nucleoside analogues zidovudine, stavudine, lamivudine and acyclovir inhibited the high- and low- affinity components of microsomal UDP-GlcUA
uptake by 65% to 80%, and 45% to 65%, respectively. When added at a concentration of
5 mM, zidovudine essentially abolished the high affinity uptake pathway (>95% inhibition),
and inhibited the low affinity pathway by >65%. Consistent with previous reports (Battaglia
et al. 1996) relating to UDP-GlcUA uptake by rat liver microsomes, probenecid and
furosemide inhibited both components of microsomal UDP-GlcUA uptake. Glucuronide
conjugates variably inhibited both the high and low affinity components of microsomal UDP-
GlcUA uptake (by 10 to 40%). In contrast to all other compounds, which caused greater
inhibition of the high-affinity microsomal UDP-GlcUA pathway compared to the low affinity
pathway, 4-MU glucuronide and zidovudine glucuronide caused equal or greater inhibition of
the low affinity pathway, implying that for some glucuronide conjugates this may be the
preferable transport process.

As many of the drugs screened as potential inhibitors of microsomal UDP-GlcUA uptake are
substrates for glucuronidation (a process that utilizes UDP-GlcUA), the presence of
glucuronide conjugates in unfiltered incubation samples was assessed to exclude UDP-GlcUA
utilization through glucuronidation of the aglycone (i.e. inhibitor) as a potential cofounding
factor in inhibition experiments. This was achieved by screening mass spectra from
incubations obtained in the presence of potential inhibitors for the presence of ‘peaks’ at m/z
values corresponding to the glucuronide conjugates of the potential uptake inhibitor. Where
available, data were confirmed by the use of authentic glucuronide standards. Under the
incubation conditions employed to assess the kinetics of microsomal UDP-GlcUA uptake, the
formation of glucuronide conjugates was not observed for any of the potential inhibitors. The
capacity of this approach to detect the presence of glucuronide conjugates was confirmed by
control experiments using validated in vitro metabolism incubation conditions for each
potential inhibitor, and with the use of authentic glucuronide standards.
Discussion

The kinetics of microsomal UDP-GlcUA uptake by human liver microsomes are reported here for the first time. Two components with distinct, saturable kinetic behavior were observed for human liver microsomal UDP-GlcUA uptake (Figure 2). The binding affinities of the two components differed by more than an order of magnitude; mean (±S.D) $K_d$ values for the high- and low- affinity components were 13±6µM and 374±175µM, respectively. By contrast, the maximal rates of uptake for the two components were relatively similar. Mean $V_{\text{max}}$ values for the high- and low- affinity components differed by only 2.3-fold; 56 ±26pmol/min/mg and 131±35pmol/min/mg, respectively (Table 1). $U_{\text{int}}$ for the high affinity component was an order of magnitude higher compared to the low affinity component. These data are consistent with previous reports of bimodal kinetics for UDP-GlcUA uptake by conditioned rat liver microsomes (Battaglia et al 1996; Kobayashi et al 2006). $K_d$ values obtained in the current study are comparable those of Kobayashi et al (2006) (23 and 104µM), but differ by an order of magnitude to those of Battaglia et al. (1996) (1.6±0.4 µM and 38 ±7µM). Based on model fitting, and consistent with the known existence of multiple UDP-sugar transporters, it is assumed that the two components of microsomal UDP-GlcUA uptake result from the involvement of at least two transport proteins, although the involvement of a single transport protein with multiple substrate binding sites cannot be discounted. Taken together, these data indicate that related transport processes may be involved in microsomal UDP-GlcUA uptake in humans and rats. This observation is consistent with the critical and presumably conserved nature of this process.

In order to characterize the transporter mediated characteristics of microsomal UDP-GlcUA uptake and exclude confounding processes such as the non-specific binding of UDP-GlcUA to the microsomal matrix, experiments were performed in the presence of protein ($N$-ethylmaleimide) and membrane (alamethacin) modifying agents. Treatment of HLM with the pore forming peptide alamethacin is now routine practice in glucuronidation kinetic
experiments when HLM are used as the enzyme source (Fisher et al. 2000; Boase and Miners 2002). This agent disrupts the integrity of the microsomal membrane (Schwarz et al. 1987), thereby facilitating UDP-GlcUA transfer into (and out of) the lumen. The absence of UDP-GlcUA in washed HLM samples treated with alamethacin confirms that the accumulation of UDP-GlcUA in microsomal uptake experiments does not result from non-specific binding to the microsomal matrix, and that the reported uptake kinetics reflects a true internalization of the UDP-GlcUA within HLM. The lack of non-specific binding of UDP-GlcUA to HLM was confirmed by equilibrium dialysis (data not shown). Modification of cysteine residues by alkylation with \(N\)-ethylmaleimide is a well characterized approach for disrupting protein function (Guidotti and Konigsberg 1964). The absence of UDP-GlcUA uptake in the presence of \(N\)-ethylmaleimide confirmed that UDP-GlcUA uptake by HLM is a protein mediated process. Both the low- and high- affinity components of UDP-GlcUA uptake were essentially abolished (<5% control activity) by \(N\)-ethylmaleimide. Taken together, these data confirm that microsomal UDP-GlcUA uptake is a transporter mediated process and indicate that multiple transporter proteins are involved in this process.

Bossuyt and Blankaert (1994a and 1995) characterised the trans-stimulation of microsomal UDP-GlcUA by related uridine sugars in rat liver microsomes and proposed a model whereby the uptake of UDP-GlcUA is coupled to UDP-GlcNAc and uridine monophosphate (UMP) uptake and efflux. The requirement for HLM to be pre-loaded with UDP-GlcNAc in the current study confirms that the uptake of UDP-GlcUA by HLM is linked (either directly or indirectly) to efflux (or utilization) of UDP-GlcNAc. Notably, while trans-stimulation of UDP-GlcUA uptake was achieved by pre-loading HLM with UDP-GlcNAc and washing, co-incubation of UDP-GlcUA with either UDP-GlcNAc or UDP-Glc inhibited both the high- and low- affinity components of microsomal UDP-GlcUA uptake by 40 to 80%. These data suggest a possible lack of directionality or coupling of UDP-GlcUA and UDP-GlcNAc transport, and it is conceivable that the trans-stimulation of microsomal UDP-GlcUA uptake
may result from coupled transport with UMP. In this regard, it is plausible that conditioning of microsomes with an alternate UDP-sugar (i.e. UDP-GlcNAc) may stimulate UDP-GlcUA uptake by increasing the intracellular UMP pool rather than a direct interaction with the transport protein during the incubation. Further research that directly quantifies the intra- and extra-cellular concentration-time profile for each of these compounds is required to fully elucidate the mechanism of this process.

Previous studies in alternate systems (rat liver microsomes and recombinant proteins) have routinely utilized a 0.45µm pore-size mixed cellulose membrane filter to separate microsomes following uptake experiments (Bossuyt and Blanckaert 1994, 1995, 1997 and 2001, Kobayashi et al. 2006). However, morphological studies of microsomes prepared from human liver have demonstrated that intact microsomes typically range in diameter from 0.05 to 0.5µm (Palade and Siekevitz 1956). As such, the use of 0.45µm pore-size membrane filters was considered inappropriate for studying microsomal UDP-GlcUA uptake in the current study. Indeed, it was demonstrated that the use of 0.45µm pore-size membrane filters ‘trapped’ only 44% of added protein, while the use of 0.025µm pore-size membrane filters trapped >95% of added protein. Subsequent activity assays confirmed that the protein lost when filtering with 0.45µm pore-size membrane filters was due to loss of HLM. Additionally, previous studies have typically employed a non-selective radioisotopic technique to quantify intra-cellular UDP-GlcUA, whereby radioisotope count rather than UDP-GlcUA is quantified. Given the utilization and potential hydrolysis of UDP-GlcUA in microsomal samples, the quantification of UDP-GlcUA by this technique may be confounded by breakdown products or metabolites that retain the radioisotopic label. In the current study, UDP-GlcUA was directly measured at the m/z value of the base compound in negative [M-H] mode (578.0186), thereby ensuring that only intracellular UDP-GlcUA was quantified. As the alternate UDP-sugars (UDP-Glc and UDP-GlcNAc) utilized in the current study differ in terms of molecular mass, due to the presence of different sugar moieties, the direct measurement of UDP-GlcUA
at the m/z value of the base compound (578.0186) ensures that these data are not affected by alternate UDP-sugars or by breakdown products. It is plausible that experimental artifacts may have contributed to the variability in previous reports of microsomal UDP-GlcUA uptake (e.g. by rat liver microsomes).

Studies were undertaken to assess the potential inhibition of human liver microsomal UDP-GlcUA uptake by a panel of drugs, alternate UDP-sugars and glucuronides (Figure 3). Glucuronide conjugates inhibited the high- and low-affinity components of UDP-GlcUA uptake by 15 to 40%, and 10 to 20%, respectively. The alternate UDP sugars UDP-Glc and UDP-GlcNAc inhibited UDP-GlcUA uptake by 70 and 80% (high affinity component), and 40 and 55% (low affinity component), respectively. When added at a concentration of 100μM, the nucleoside analogs zidovudine, stavudine, lamivudine and acyclovir inhibited the high- and low-affinity components of UDP-GlcUA uptake by 45 to 65% and 65 to 80%, respectively. Further, 5mM zidovudine essentially abolished the high affinity uptake pathway (>95% inhibition). Drugs known to inhibit SLC35 transport proteins inhibited UDP-GlcUA uptake by 40 to 80%, further supporting the hypothesis that the transporters involved in UDP-GlcUA uptake by HLM are members of this family of transport proteins. Several of the nucleotide reverse transcriptase inhibitors and anion transport inhibitors that were screened as potential inhibitors of microsomal UDP-GlcUA uptake are substrates for glucuronidation, a process that utilizes UDP-GlcUA as the cofactor. As such, the utilization of UDP-GlcUA through glucuronidation of the aglycone (inhibitor) was excluded as a potential confounding factor by quantifying the presence of glucuronide conjugates of the aglycone in unfiltered incubation samples.

Data presented here support the hypothesis that microsomal UDP-GlcUA uptake occurs via a protein-mediated pathway. In this regard, it is generally believed that protein(s) of the solute carrier (SLC) group of membrane transport proteins serve as luminal UDP-GlcUA uptake
transporters (Kobayashi et al. 2006). Indeed, UDP-GlcUA transport has been demonstrated for two recombinant proteins, SLC35B1 (UGTRel1) and SLC35D1 (UGTRel7), expressed in V79 cells (Muraoka et al. 2001). Further, SLC35D1 is known to be expressed in the ER membrane (Bakker et al. 2009). Despite limited data regarding this family of transporters, there is sufficient evidence to support the hypothesis that one or more SLC35 transporters may be responsible for the uptake of UDP-GlcUA into the ER lumen. It has previously been proposed that homo- (with other UGT) and hetero- (with non-UGT proteins) dimerization of UGT results in the formation of hydrophilic pores in the ER membrane that facilitate the passive diffusion of UDP-GlcUA into the ER lumen (Ikushiro et al. 1997 and 1999, Radominska-Pandya et al. 1999). However, it is now generally believed that discrete transport proteins are responsible for UDP-sugar translocation across the microsomal membrane (see previous discussion).

In conclusion, this study has demonstrated that human liver microsomal UDP-GlcUA uptake is a protein (transporter) mediated process with high- and low- affinity components. Given the importance of glucuronidation as a clearance and detoxification pathway for a myriad of endogenous and exogenous chemicals including drugs from most therapeutic classes, and the pivotal involvement of microsomal UDP-GlcUA uptake in the glucuronidation pathway, further studies are warranted to unambiguously identify the transporters responsible. Moreover, data presented here raise the intriguing possibility that impaired glucuronidation of exogenous and endogenous compounds could result from inhibition of UDP-GlcUA uptake into the ER membrane by drugs and other compounds.
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Author Contributions

Participated in research design: Rowland, Mackenzie, Miners

Conducted experiments: Rowland,

Contributed new reagents or analytic tools: Rowland

Performed data analysis: Rowland, Miners

Wrote or contributed to the writing of the manuscript: Rowland, Mackenzie, Miners
References


Footnotes

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Figures

**Figure 1.** Mass spectrum for an incubation sample containing UDP-GlcUA.

**Figure 2.** Representative velocity versus substrate concentration (A) and Eadie-Hofstee (B) plots (HLM H40) for UDP-GlcUA uptake by HLM. Points are experimentally determined values, whereas curves are from model-fitting. Goodness of fit parameters from fitting with equation 1 were: $R^2 = 0.999$, $F$ statistic $= 2870$, and percent standard error of parameter fit for the high and low affinity $k_d$ values $= 15\%$ and $23\%$, respectively.

**Figure 3.** Effect of alternate UDP-sugars, anion transport inhibitors, nucleotide reverse transcriptase inhibitors and glucuronide conjugates on microsomal UDP-GlcUA uptake. Each bar represents the mean of quadruplicate measurements.
### Table 1 – Kinetic parameters for UDP-GlcUA uptake by HLM

<table>
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<th>Liver</th>
<th>K&lt;sub&gt;d1&lt;/sub&gt;</th>
<th>V&lt;sub&gt;max1&lt;/sub&gt;</th>
<th>U&lt;sub&gt;int1&lt;/sub&gt;</th>
<th>K&lt;sub&gt;d2&lt;/sub&gt;</th>
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<td>151</td>
<td>0.27</td>
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</table>

| Mean  | 13            | 56              | 4.6             | 374           | 131             | 0.41            |
| SD    | 5.9           | 26              | 1.7             | 175           | 35              | 0.17            |

Units: K<sub>d</sub>: μM, V<sub>max</sub>: pmol/min/mg, U<sub>int</sub>: μL/min/mg
Figure 2

A

B

Rate (pmol/min/mg) vs [UDPGA] - (μM)

Rate (pmol/min/mg) / [UDPGA] - (μM)
Figure 3

% Inhibition (±S.D.)

- 2.5 μM UDP-GlcUA
- 1000 μM UDP-GlcUA


Categories:
- UDP-Sugars
- Nucleoside Analogs
- Anion Transport Inhibitors
- Glucuronide Conjugates