Transporter-Mediated Uptake of UDP–Glucuronic Acid by Human Liver Microsomes: Assay Conditions, Kinetics, and Inhibition

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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 ± 6 vs. 374 ± 175 µM), but were comparable in terms of the maximal rate of uptake, with mean \( V_{\text{max}} \) values differing less than 2.3-fold (56 vs. 131 pmol/min per mg). Variability in total intrinsic transporter activity (\( U_{\text{trans}} \) 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 preloading (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 glucuronic conjugates, at low (2.5 µM) and high (1000 nM) 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 100 µM. 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 endoplasmic reticulum 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, nonsteroidal 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-glucuronosyltransferase (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.

Although 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 Blanckaert, 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 (Bossuyt and Blanckaert, 1994a; Berg et al., 1995). 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 Blanckaert, 1995). Kinetic studies with rat liver microsomal 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, 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 used suboptimal assay conditions and a nonselective radioisotopic filtration technique. Accordingly, previous studies have produced conflicting results regarding luminal UDP-GlcUA uptake (Battaglia et al., 1996; Murao et al., 2001; Kobayashi et al., 2006). Thus, this study sought to develop and validate a sensitive and specific

ABBREVIATIONS: ER, endoplasmic reticulum; HLM, human liver microsome; 4-MU, 4-methylumbelliferone; UDP–Glc, UDP-glucose; UDP–GlcNAc, UDP-N-acetylglucosamine; UDP–GlcUA, UDP–glucuronic acid; UGT, UDP-glucuronosyltransferase; UMP, uridine monophosphate; UPLC-MS, ultra-high-performance liquid chromatography–mass spectrometry.
analyzed by UPLC-MS. The effects of microsomal conditioning, freeze-thawing
were immediately filtered through a presoaked (transport buffer) MF-membrane
mixed, and centrifuged (6000
were set at 225
C and 90
C for 90 minutes in transport buffer
prior to uptake experiments, HLM were conditioned by incubating at 37°C for 90 minutes in transport buffer [phosphate buffer (pH 7.4) containing sucrose (250 mM), potassium chloride (150 mM), and magnesium chloride (5 mM)], then preloaded with UDP-GlcNAc by incubating for a further 90 minutes at 37°C in the presence of UDP-GlcNAc (2 mM). The conditioned HLM pellet was separated by centrifugation (30,000 g) and resuspended in transport buffer. The kinetics of microsomal UDP-GlcUA uptake was determined according to the method of Lowry et al. (1951).

Kinetcis of Microsomal UDP-GlcUA Uptake. Prior to uptake experiments, HLM were conditioned by incubating at 37°C for 90 minutes in transport buffer [phosphate buffer (20 mM, pH 7.4) containing sucrose (250 mM), potassium chloride (150 mM), and magnesium chloride (5 mM)], then preloaded with UDP-GlcNAc by incubating for a further 90 minutes at 37°C in the presence of UDP-GlcNAc (2 mM). The conditioned HLM pellet was separated by centrifugation (30,000g) 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 (200 µl) contained conditioned HLM (5 mg/ml) and NAD+ (2 mM) in transport buffer. Incubations were initiated by the addition of UDP-GlcUA (5–1000 µM) and continued for 10 minutes at 37°C in a shaking water bath. Following incubation, samples were immediately filtered through a presoaked (transport buffer) MF-membrane filter (0.025-
 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 were undertaken to 1) establish an assay and optimize conditions for the reliable measurement of UDP-GlcUA uptake by HLM, 2) characterize the kinetics of UDP-GlcUA uptake by HLM from individual donors and assess the interindividual variability in this process, and 3) 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 nonhuman 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 is shown that both components may be stimulated by preincubation of HLM with alternate UDP-sugars, or inhibited to a varying extent by coinubation in the presence of alternate UDP-sugars, drugs, and glucuronide conjugates.

Materials and Methods

4-Methylumbelliferone glucuronide, acetyl-carnosine, N-acetylcarnosine, presoaked MF-membrane, probenecid, stauvudine, UDP-glucose (UDP-Glc), UDP-GlcUA, UDP-GlcNAc, zidovudine, and zidovudine glucuronide were obtained from Sigma-Aldrich (Sydney, Australia). Acetaminophen glucuronide, 1-hydroxypropylene glucuronide, morphine 3-glucuronide, phenolphthalein glucuronide, propofol glucuronide, SN-38 glucuronide, and zidovudine glucuronide (100 µM) to inhibit microsomal UDP-GlcUA uptake was evaluated by coinubation with these compounds. Potential inhibitors were added to incubations at the same concentrations as UDP-GlcUA, thereby representing cytosolic (cis)-addition. Microsomal uptake of UDP-GlcUA in the presence of the potential inhibitors was assessed at low (2.5 µM) and high (1000 µM) 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 dimethylsulfoxide (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 (2000 µM).

Non-specific Binding. To exclude confounding nonspecific binding of UDP-Glc-UA to HLM, binding of UDP-Glc-UA to a pool (n = 12) of HLM was measured by equilibrium dialysis according to the method of McElre 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 a Sigma-Aldrich dialysis membrane (molecular mass cutoff, 12 kDa). One side of the dialysis cell was loaded with 1 ml of UDP-Glc-UA (5–1000 µM) in phosphate buffer (0.1 M, pH 7.4). The other compartment was loaded with 1 ml of HLM (5 mg/ml) in phosphate buffer (0.1 M, pH 7.4). The dialysis cell assembly was immersed in a water bath maintained at 37°C and rotated at 12 rpm for 4 hours. Control experiments were performed with phosphate buffer or HLM on both sides of the dialysis cell to allow high and low concentrations of UDP-Glc-UA to ensure that equilibrium was attained. A 200-µl aliquot was collected from each compartment, treated with ice-cold methanol containing hydrochloric acid (0.1%; 200 µl), and cooled on ice. Samples were subsequently centrifuged at 4000g for 10 minutes at 10°C, and an aliquot of the supernatant fraction (5 µl) was analyzed by UPLC-MS. The binding of UDP-Glc-UA to HLM was calculated as the concentration of UDP-Glc-UA in the buffer compartment divided by the concentration of UDP-Glc-UA 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 × 150 mm, 1.8-µm particle size) analytical column (Waters Corporation, Sydney, Australia). UDP-Glc-UA was separated from matrix components using a gradient mobile phase comprising 10 mM ammonium acetate (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.2 ml/min. The elution gradient was increased linearly from 2.5% B to 90% B over 3 minutes. The total run time, including reconditioning of the column to initial conditions, was 5 minutes. The retention time for UDP-Glc-UA was 1.49 minutes. Column eluant was monitored by mass spectrometry, performed on a Waters Q-ToF Premier quadrupole, orthogonal acceleration time-of-flight tandem mass spectrometer (Waters Corporation) operated in negative ion mode with electrospray ionization (Fig. 1). The nebulizer and cone gases were set to 578. 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 3 eV. Resulting mass spectra were analyzed using Waters QuanLynx software (Waters Corporation). UDP-Glc-UA was detected at an m/z of 578.0186 [M-H] (Fig. 1). The lower limit of quantification for UDP-Glc-UA, defined as 10 times the background noise, was 60 nM (0.1 µg/l). The linear detection range for UDP-Glc-UA was 0.06–100 µM. This range spanned from 8-fold lower than the minimum concentration observed

UDP-GlcUA uptake by HLM was linear with respect to incubation time of 15 minutes and HLM concentration of 10 mg/ml.
Kinetics and Inhibition of UDP-GlcUA Uptake by HLM

4-Methylumbelliferone Glucuronidation Activity and Detection of Glucuronide Conjugates. The capacity of filtered and unfiltered HLM samples to catalyze the glucuronidation of 4-methylumbelliferone (4-MU) was assessed 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 HSS T3 C18 (2.1 × 150 mm, 1.8-μm particle size) analytical column (Waters Corporation). Glucuronide conjugates were separated from matrix components using a gradient mobile phase comprising 10 mM ammonium formate (adjusted to pH 3.0 with formic acid; mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.25 ml/min. The elution gradient was increased linearly from 5% B to 75% B over 5 minutes. The total run time, including reconditioning of the column to initial conditions, was 7 minutes. 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 400 and 50 l/h, respectively. Desolvation and source temperature were set at 280°C and 90°C, respectively. The capillary and cone voltages were set to 2600 and 50 V, respectively. Time-of-flight data were collected in MS mode, with data collected between m/z 100 and 1000 Da at a collision energy of 5 eV.

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

\[
V = \frac{V_{\text{max1}}}{K_{d1} + [S]} + \frac{V_{\text{max2}}}{K_{d2} + [S]}
\]  

where \( V \) is the rate of uptake, \( V_{\text{max}} \) is the maximum rate of uptake attributed to the \( n \)th transporter mode, \( K_{d} \) is the dissociation constant for the \( n \)th transporter mode, and [S] is the total substrate concentration. Intrinsic transporter activity (\( U_{\text{intrinsic}} \)) was calculated as \( \frac{V_{\text{max}}}{K_{\text{d}}} \). 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 Fig. 3 are the mean (±S.D.) 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 (5 mg/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 0.45-μm 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 0.45-μm membranes exhibited 4-MU glucuronidation activity comparable to unfiltered samples; \( K_{d} \) and \( V_{\text{max}} \) values from unfiltered, 0.22-μm filtered, and 0.45-μm filtered samples were 80 μM and 69,500 pmol/min per mg, 78 μM and 71,700 pmol/min per mg, and 82 μM and 68,300 pmol/min per mg, respectively. Negligible 4-MU glucuronidation activity was observed in the samples filtered through a 0.025-μm 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 nonspecific 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 5 ml of ice-cold transport buffer was sufficient to remove all noneentalized UDP-GlcUA from the reservoir compartment in the presence of incubation samples. Equilibrium dialysis experiments confirmed that nonspecific binding of UDP-GlcUA to pooled HLM (5 mg/ml) was negligible (<15%) across the UDP-GlcUA concentration range of 5–1000 μM. 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, 1997; Muraoka...
et al., 2001), UDP-GlcUA uptake was reduced by 63 ± 2% when HLM were not preloaded with UDP-GlcNAc. This effect was independent of UDP-GlcUA concentration across the range 50–1000 μM. 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 (0–4 cycles) caused a reduction in microsomal UDP-GlcUA uptake due to decreases in both affinity (increased $K_d$) and capacity (decreased $V_{\text{max}}$). 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 17 μM and from 348 to 487 μM, respectively. The magnitude of the effect was proportional to the number of freeze-thaw cycles: a 40% reduction in total $U_{\text{int}}$ for UDP-GlcUA uptake was observed following two freeze-thaw cycles (4.9 vs. 6.9 μmol/min per mg), whereas a 70% reduction in total $U_{\text{int}}$ was observed following four freeze-thaw cycles (4.9 vs. 8.3 μmol/min per mg).

A decrease in UDP-GlcUA concentration was also observed in unfiltered samples that were incubated at 37°C for 10 minutes in the absence of NAD$^+$. This decrease was concentration-dependent, ranging from 80 to 25% at UDP-GlcUA concentrations of 50 and 1000 μM, respectively. No decrease in UDP-GlcUA concentration was observed for unfiltered incubation samples in the presence of NAD$^+$ (2 mM). As such, 2 mM 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 350 μM (Ishii et al., 2012). The presence of NAD$^+$ (2 mM) 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 (Fig. 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 ± 5.9 and 374 ± 175 μM, respectively. Notably, interliver variability in the affinity for both components was relatively minor (<5-fold), with $K_d$ values ranging from 5.1 to 23 μM and 139 to 610 μM for the high- and low-affinity components, respectively. Mean (±S.D.) $V_{\text{max}}$ values for the high- and low-affinity components were 56 ± 26 and 131 ± 35 pmol/min per mg, respectively. $U_{\text{int}}$ was an order of magnitude higher for the high-affinity component of microsomal UDP-GlcUA uptake. As with binding affinity, interliver variability in capacity of the two components was minor (<5-fold), with $V_{\text{max}}$ values ranging from 18 to 92 pmol/min per mg and 86 to 196 pmol/min per mg for the high- and low-affinity components, respectively.

**Inhibition of Microsomal UDP-GlcUA Uptake.** The ability of alternate UDP-sugars, transporter inhibitors (reported inhibitors of SLC35 transporter proteins), nucleotide reverse-transcriptase inhibitors, and glucuronide conjugates (100 μM) to inhibit UDP-GlcUA uptake was assessed at low (2.5 μM) and high (1000 μM) UDP-GlcUA concentrations (Fig. 3). Substitution of the mean kinetic parameters given in Table 1 into eq. 1 (Data Analysis) indicates that the high-affinity component is responsible for 90% of uptake at a UDP-GlcUA concentration of 2.5 μM, whereas the low-affinity component is responsible for ~70% of uptake at a UDP-GlcUA concentration of 1000 μM. 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 100 μM, zidovudine essentially abolished the high-affinity 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 10–40%). In contrast to all other compounds, which caused greater inhibition of the high-affinity microsomal UDP-GlcUA pathway compared with 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 confounding 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 used 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.

**Fig. 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 eq. 1 were as follows: $R^2 = 0.999$, $F$ statistic = 2870, and percent standard error of parameter fit for the high- and low-affinity $k_d = 15$% and 23%, respectively.
Discussion

This study is the first to report the kinetics of microsomal UDP-GlcUA uptake by human liver microsomes. Two components with distinct, saturable kinetic behavior were observed for human liver microsomal UDP-GlcUA uptake (Fig. 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 ± 6 and 374 ± 175 $\mu$M, 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 ± 26 and 131 ± 35 pmol/min per mg, respectively (Table 1).

$U_{\text{int}}$ for the high-affinity component was an order of magnitude higher compared with 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 with those of Kobayashi et al. (2006) (23 and 104 $\mu$M), but differ by an order of magnitude compared to those of Battaglia et al. (1996) (1.6 ± 0.4 and 38 ± 7$\mu$M). 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.

To characterize the transporter-mediated characteristics of microsomal UDP-GlcUA uptake and exclude confounding processes such as the nonspecific 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 nonspecific binding to the microsomal matrix, and that the reported uptake kinetics reflect a true internalization of the UDP-GlcUA within HLM. The lack of nonspecific binding of UDP-GlcUA to HLM was confirmed by equilibrium dialysis (data not shown). Modification of

### Table 1

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<th>$V_{\text{max}}$</th>
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</table>

Fig. 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.
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 Blanckaert (1994a, 1995) characterized 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 preloaded 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, whereas trans-stimulation of UDP-GlcUA uptake was achieved by preloading HLM with UDP-GlcNAc and washing, coincubation of UDP-GlcUA with either UDP-GlcNAc or UDP-Glc inhibited both the high- and low-affinity components of microsomal UDP-GlcUA uptake by 40–80%. 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 extracellular 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 used a 0.45-μm pore size mixed cellulose membrane filter to separate microsomes following uptake experiments (Bossuyt and Blanckaert, 1994a and b, 1995, 1997; Kobayashi et al., 2006). However, morphologic studies of microsomes prepared from human liver have demonstrated that intact microsomes typically range in diameter from 0.05 to 0.5 μm (Palade and Siekevitz, 1956). As such, the use of a 0.45-μm 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 0.45-μm pore size membrane filters “trapped” only 44% of added protein, whereas the use of 0.025-μm pore size membrane filters trapped >95% of added protein. Subsequent activity assays confirmed that the protein lost when filtering with 0.45-μm pore size membrane filters was due to loss of HLM. Additionally, previous studies have typically used a noneselective radioisotopic technique to quantify intracellular 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) used 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 (Fig. 3). Glucuronide conjugates inhibited the high- and low-affinity components of UDP-GlcUA uptake by 15–40% and 10–20%, 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 100 μM, the nucleoside analogs zidovudine, stavudine, lamivudine, and acyclovir inhibited the high- and low-affinity components of UDP-GlcUA uptake by 45–65% and 65–80%, respectively. Further, 5 mM zidovudine essentially abolished the high-affinity uptake pathway (>95% inhibition). Drugs known to inhibit SLC35 transport proteins inhibited UDP-GlcUA uptake by 40–80%, 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 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 proteins) and hetero-dimerization (with non-UGT proteins) 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 (Ishishiro, 1997, 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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Authorship Contributions

Participated in research design: Rowland, Mackenzie, Miners.

Conducted experiments: Rowland.

Contributed new reagents or analytical tools: Rowland.

Performed data analysis: Rowland, Miners.

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References


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