Drug and Chemical Glucosidation by Control Supersomes and Membranes from Spodoptera frugiperda (Sf) 9 Cells: Implications for the Apparent Glucuronidation of Xenobiotics by UDP-glucuronosyltransferase 1A5

Nuy Chau, Leyla Kaya, Benjamin C. Lewis, Peter I. Mackenzie, and John O. Miners

Department of Clinical Pharmacology (N.C., L.K., B.C.L., P.I.M., J.O.M.) and Flinders Centre for Innovation in Cancer (B.C.L., P.I.M., J.O.M.), Flinders University College of Medicine and Public Health, Adelaide, Australia

Received October 21, 2018; accepted November 29, 2018

ABSTRACT

Accumulating evidence indicates that several human UDP-glucuronosyltransferase (UGT) enzymes catalyze both glucuronidation and glucosidation reactions. Baculovirus-infected insect cells [Trichoplusia ni and Spodoptera frugiperda (Sf9)] are used widely for the expression of recombinant human UGT enzymes. Following the observation that control Supersomes (c-SUP) express a native enzyme capable of glucosidating morphine, we characterized the glucosidation of a series of aglycones with a hydroxyl (aliphatic or phenolic), carboxylic acid, or amine functional group by c-SUP and membranes from uninfected Sf9 cells. Although both enzyme sources glucosidated the phenolic substrates investigated, albeit with differing activities, differences were observed in the selectivities of the native UDP-glucosyltransferases toward aliphatic alcohols, carboxylic acids, and amines. For example, zidovudine was solely glucosidated by c-SUP. By contrast, c-SUP lacked activity toward the amines lamotrigine and trifluoperazine and did not form the acyl glucoside of mycophenolic acid, reactions all catalyzed by uninfected Sf9 membranes. Glucosidation intrinsic clearances were high for several substrates, notably 1-hydroxypyrene (~1400–1900 µl/min mg). The results underscore the importance of including control cell membranes in the investigation of drug and chemical glucuronidation by UGT enzymes expressed in T. ni (High-Five) and Sf9 cells. In a coincident study, we observed that UGT1A5 expressed in Sf9, human embryonic kidney 293T, and COS7 cells lacked glucuronidation activity toward prototypic phenolic substrates. However, Sf9 cells expressing UGT1A5 glucosidated 1-hydroxypyrene with UDP-glucuronic acid as the cofactor, presumably due to the presence of UDP-glucose as an impurity. Artificial glucosidation may explain, at least in part, a previous report of phenolic glucuronidation by UGT1A5.

Introduction

Enzymes of the UDP-glucuronosyltransferase (UGT) family play a pivotal role in the clearance and detoxification of a structurally diverse range of substrates that include drugs, nondrug xenobiotics, and endogenous compounds. The 19 human UGT proteins classified in subfamilies 1A, 2A, and 2B primarily catalyze the transfer of glucuronic acid from the cofactor UDP-glucuronic acid (UDP-GlcUA) to a typically lipophilic substrate (or aglycone) bearing a nucleophilic “acceptor” functional group to form a glucuronide conjugate that is excreted in urine and/or bile (Mackenzie et al., 2005; Miners et al., 2010). By contrast, UGT 3A1 and 3A2 use UDP-sugars other than UDP-GlcUA [e.g., UDP-glucose (UDP-Glc), UDP-xyllose, and UDP-N-acetylgalactosamine] as the cofactor (Mackenzie et al., 2008, 2011). Although glucuronidation is the major metabolic pathway mediated by UGT1A1, 2A, and 2B enzymes, 1A and 2B subfamily enzymes may additionally use sugar donors other than UDP-GlcUA, especially UDP-Glc. In particular, UGT2B7 catalyzes both the glucuronidation and glucosidation of a number of substrates, including morphine (MOR), forming phenolic-, acyl- and N-glucosides (Mackenzie et al., 2003; Tang et al., 2003; Toide et al., 2004; Buchheit et al., 2011; Chau et al., 2014). At least with MOR glycosidation by UGT2B7, glucuronidation predominates over glucosidation because the binding affinity of UDP-GlcUA is higher than that of UDP-Glc (Chau et al., 2014). Substrates of other UGT enzymes (e.g., 1A1, 1A9, and 2B10) have also been reported to form glycoside conjugates other than glucuronides (Fevery et al., 1977; Senafi et al., 1994; Lu et al., 2018; Chau and Miners, unpublished data). Despite the likelihood that UGT-catalyzed glucuronidation and glucosidation of xenobiotics may occur as complementary metabolic pathways, glucosidation has received little attention (Tang, 1990; Meech et al., 2012).

It is well established that the individual human UGT enzymes exhibit distinct, but frequently overlapping, substrate and inhibitor selectivities (Miners et al., 2004, 2010). In this regard, the availability of cDNA-expressed UGT proteins has been pivotal in the characterization of UGT function. Recombinant UGTs have been expressed in numerous
mammalian and nonmammalian cell lines (Radominska-Pandya et al., 2005). Examples include COS (African green monkey kidney fibroblasts), human embryonic kidney 293T cell line (HEK293T), V79 (Chinese hamster lung fibroblasts), yeast (Pichia pastoris and Saccharomyces cerevisiae), and baculovirus-infected insect cells [Spodoptera frugiperda (Sf9) and Trichoplusia ni] (e.g., Fournel-Gigleux et al., 1991; Jin et al., 1997; Nguyen and Tukey, 1997; Ouzine et al., 1999; Uchaipichat et al., 2004; Zhang et al., 2012). Of these, the use of commercially available UGT-expressing Supersomes, prepared from baculovirus-infected T. ni cells, has become widespread in academia and industry. However, in a recent study of the comparative 3- glucuronidation and glucosidation of MOR by UGT2B7, we observed that Supersomes expressing UGT2B4, 2B15, and 2B17 protein as well as control Supersomes [c-SUP; insect cell “control” microsomes prepared from T. ni (High-Five) cells infected with wild-type baculovirus] all exhibited significant and comparable MOR 3-glucosidation activities (Chau et al., 2014).

Since these data indicate that Supersomes express a “native” enzyme capable of MOR 3-glucuronidation, we characterized the glucosidation of a series of aglycones with a phenolic [1-hydroxypyrene (1-OHP), 4-methylumbelliferone (4-MU), MPA, mycophenolic acid (MPA), 1-naphthol (1-NAP), and 4-nitrophenol (4-NP)], aliphatic alcohol [codeine (COD), 21-hydroxyprogestrone (21-OHP)], phenethyl alcohol (PE), and zidovudine (AZT), acyl [MPA, S-naproxen (S-NAP)], or amine [benzocaine (BZC), lamotrigine (LTG), and trifluoperazine (TFP)] acceptor functional group (see Supplemental Fig. 1 for structures and sites of conjugation) by c-SUP. The glucosidation of these aglycones was additionally characterized using the enriched membrane fraction from uninfected Sf9 cells (subsequently referred to as “Sf9 membranes”) since baculovirus-infected Sf9 cells are also used for UGT expression (e.g., Zhang et al., 2012) and are available commercially as Baculosomes.

Coincident with these studies, we conducted an investigation of UGT1A5 structure function. UGT1A5 expressed in baculovirus-infected Sf9 cells has been reported to glucuronidate a number of phenolic substrates, including 1-OHP and 4-MU (Finel et al., 2005). However, we found that UGT1A5 lacked glucuronidation activity when expressed in COS7, HEK293T, and baculovirus-infected Sf9 cells. The glucuronidation activity studies reported here indicate that 1-OHP, 4-MU, and most other aglycones investigated are glucuronidated by c-SUP and/or Sf9 cell membranes. It is possible that artifactual glucuronidation by Sf9 membranes may contribute, at least in part, to the differing UGT1A5 glucuronidation data reported between laboratories.

**Materials and Methods**

**Materials**

AZT, AZT β-D-glucuronide, COD, gentamicin, 21-OHP, 1-OHP, kanamycin, 4-MU, 4-MU β-D-glucoside, 1-NAP, 1-NAP β-D-glucuronide, S-NAP, 4-NP, 1-octanolsulfonic acid sodium salt, tetracycline, TFP, triethylamine, UDP-Glc (disodium salt), and UDP-GlcUA (trisodium salt) were purchased from Sigma-Aldrich (Sydney, NSW, Australia); BZC, COD β-D-glucuronide, 1-OHP β-D-glucuronide, MPA, MPA acyl β-D-glucoside, MPA phenolic β-D-glucoside, PE, and PE β-D-glucoside were from Toronto Research Chemicals (Toronto, ON, Canada); 4-NP β-D-glucoside was from Molecule Limited (Dorset, UK); BZC N-glucoside was from Dalton Pharma Services (Toronto, ON, Canada); and MOR hydrochloride was from GlaxoSmithKline (Melbourne, VIC, Australia). LTG and LTG N2-glucuronide were a gift from the Wellcome Foundation Ltd. (London, UK). MOR 3-β-D-glucoside was synthesized in house as described by Chau et al. (2014). Microsomes from High-Five cells infected with “control” (wild-type) baculovirus (c-SUP) were purchased from Corning Gentest (BD Biosciences, North Ryde, NSW, Australia); uninfected Sf9 cells, penicillin-streptomycin solution (100 U/ml and 100 µg/ml), Cefetin reagent, and DLH10Bac Escherichia coli cells were from Invitrogen (Carlsbad, CA); COS7 and HEK293 cells were from American Type Culture Collection (Manassas, VA); and Hyclone SPX-Insect Cell Culture medium and heat-inactivated fetal bovine serum were from Thermo Fisher Scientific (Waltham, MA). Solvents and other reagents were of analytical reagent grade.

**Methods**

**Glucosidation Assay.** Incubations, in a total volume of 200 µl, contained phosphate buffer (0.1 M, pH 7.4 or pH 6.8 for carboxylic acid–containing substrates), MgCl2 (4 mM), uninfected Sf9 cell membranes (1 mg/ml), substrate, and UDP-Glc (5 mM). After a 5-minute preincubation at 37°C in a shaking water bath, reactions were initiated by the addition of UDP-Glc and performed for 2 hours. Reactions were terminated by the addition of perchloric acid [70% (v/v); 2 µl], acetic acid in methanol [2% (v/v); 200 µl], or acetic acid in methanol [4% (v/v); 200 µl], depending on the substrate (Supplemental Table 1), and were cooled on ice for 10 minutes. Samples were centrifuged (5000g for 10 minutes), and a 5- to 40-µl aliquot of the supernatant fraction was analyzed by high-performance liquid chromatography (HPLC). Rates of glucoside formation were measured at four different substrate concentrations (see Results). Experiments utilizing c-SUP as the enzyme source were as described for Sf9 membranes, except the incubation volume was 100 µl. Incubations were performed at least in duplicate (<5% variance between replicates). Incubations for mass spectrometry (MS) analysis followed the aforementioned protocols, except reactions were terminated by the addition of 2 volumes of MS-grade 4% acetic acid in methanol or 2% acetic acid in methanol (BZC glucosidation assay).

Incubation conditions for studies characterizing glucosidation kinetic parameters for 1-OHP, MPA (phenolic and acyl), MOR, and 4-MU with both c-SUP and uninfected Sf9 membranes as the enzyme source were as described earlier, with the following changes to protein concentrations and incubation times: 1-OHP (0.01 mg/ml, 15 minutes), MOR (1 mg/ml, 60 minutes), MPA (0.1 mg/ml, 15 minutes), and 4-MU (0.1 mg/ml, 30 minutes). Kinetic studies included 11 or 12 substrate concentrations that spanned the Km (or S50).

**Quantification of Glucoside Conjugate Formation by HPLC.** Glucoside conjugates were measured by reversed-phase HPLC using an Agilent 1100 series instrument (Agilent Technologies, Sydney, Australia) comprising an autoinjector, a quaternary solvent delivery system, and a UV detector (1200 series). Analytes were separated using varying chromatographic conditions, depending on the aglycone. Columns, mobile phases, absorbance wavelengths, precipitating agent, retention times of glucosides, detection method and wavelength, and injection volume are given in Supplemental Table 1. Glucoside formation was quantified by comparison of peak areas to those of a standard curve; authentic glucoside conjugates were available for BZC, 21-OHP, MPA, MOR, 4-MU, and 4-NP. Where the glucoside was unavailable, either the corresponding glucuronide (AZT, COD, LTG, 1-NAP, and 1-OHP) or glycine (S-NAP and TFP) was used for standard curve generation. The identity of the glucoside conjugates was confirmed by cochromatography with the authentic standard (where available) and from the m/z ratios and fragmentation patterns generated by liquid chromatography–mass spectrometry (LC-MS) (described later). Calibration curves included five concentrations, the ranges of which are given in Supplemental Table 1.

**Confirmation of Glucoside Formation by Ultraperformance Liquid Chromatography–MS.** Glucoside conjugates were separated and detected using a Waters ACQUITY Ultra Performance Liquid Chromatography (UPLC) system coupled to a Waters Micromass Q-TOF Premier mass spectrometer (Waters Corporation Micromass UK Ltd., Manchester, UK). Analytes were separated on an ACQUITY UPLC HSST3 column (1.8-µm particle size, 2.1 × 100 mm; Waters Corporation, Milford, MA). The mobile phase, delivered at a flow rate of 0.25 ml/min, consisted of two solutions (phase A, 100% MS-grade acetonitrile; phase B, 5% acetonitrile in water) mixed according to a gradient timetable. Initial conditions were 5% phase A–95% phase B held for 3 minutes followed by a linear gradient over 7 minutes to 60% phase A–40% phase B, which was held constant for 0.5 minutes. The total run time, including reconditioning of the column to initial conditions, was 12.5 minutes. The MS was operated in positive ion mode with electrospray ionization. Time-of-flight data were acquired in selected ion mode, where the first resolving quadrupole acquired mass data from m/z 100 to 1000. Collision cell energy alternated between 2 eV and a high-energy ramp (3–15 eV). The cone and desolvation gases were set to flow rates of 50 and 550 l/h, respectively; desolvation and source temperatures were 250°C and 90°C.
respectively; and capillary and cone voltages were 1800 and 25 V, respectively. MS data were collected as total ion chromatograms, with selected ion (pseudo multiple reaction monitoring) data extracted at the [M + H+] for each analyte of interest using Waters Quanlynx software (Waters Corporation).

**Construction of Recombinant Baculovirus.** The preparation of the human UGT1A5 cDNA (NM_019078) from epithelial colorectal adenocarcinoma (Caco-2) cells has been described previously by Finel et al. (2005). In expression in SF9 cells, the cDNA was subcloned into the pFastBac-HIT vector (Invitrogen) using XhoI and HindIII restriction sites, and the engineered pFast-UGT1A5-His sequence was confirmed on both strands (ABI 3130-XL DNA sequencer; Applied Biosystems, Vic Australia). Generation of recombinant Bacmid DNA was achieved by transposition of the UGT1A5 cDNA from pFastBac-HIT (1 ng) to the viral genome of DH10Bac chemically competent E. coli cells (50 μl). Recombinant Bacmid DNA was amplified (100-mL culture) and purified (Plasmid Midi Kit, Qiagen, Hilden, Germany). Polymerase chain reaction analysis of the recombinant Bacmid DNA was performed to identify the presence of UGT1A5 in the AcMNPV viral genome using the pUCM13 forward and reverse primers.

**Expression of UGT1A5 in SF9 Cells and Separation of SF9 Membranes.** SF9 cells adapted to suspension culture were grown in SFX-Insect medium supplemented with heat-inactivated fetal bovine serum (5%) and penicillin-streptomycin (1000 U and 1 mg). Cells were seeded (5 × 10⁶ cells/ml) and cultured in glass impeller spinner flasks (Belloco Glass, Inc., Vineland, NJ) at 28°C and 120 rpm (50% spinner volume) in exponential growth phase with a cell density between 1 × 10⁴ and 2.5 × 10⁴ cells/ml at greater than 95% viability. Infection optimization for UGT1A5 expression was undertaken in monolayer cultures of SF9 cells using the Cellfectin method described by the manufacturer (Invitrogen). Large-scale expression of UGT1A5 was performed in 1-L shaker flasks with SF9 cells in midlogarithmic growth at a seeding density of 1 × 10⁶ cells/ml. Cells were infected with AcMNPV-UGT1A5 at multiplicity of infection 10 (150 μL; 2 × 10⁸ pfu/ml) and harvested 48 hours postinfection by centrifugation at 850 g for 10 minutes.

The enriched membrane fraction from both SF9 cells infected with UGT1A5-containing Bacmid (i.e., expressing UGT1A5) and uninfected SF9 cells was isolated by sonication and ultracentrifugation. Pelleted cells were resuspended in cold deionized water (0.33 g pellet/ml); homogenized with 15 strokes using a Potter-Elvehjem homogenizer; sonicated by eight 1-second "bursts," each separated by 1-minute cooling on ice, using a Vibra Cell VCX 130 Ultrasonics Processor (Sonics and Materials, Newton, CT); and then centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant fraction was decanted and centrifuged at 105,000g at 4°C for 75 minutes. The pellet, which comprised the enriched membrane fraction, was resuspended in phosphate buffer (0.1 M, pH 7.4) and stored at −80°C until use.

The UGT1A5 cDNA, cloned in the pEF-IRE-6α-puro 6 vector, was stably expressed in HEK293T and COS7 cells using the procedure described by Uchaipichat et al. (2004), and cell lysates were used for immunoblotting and assessment of enzyme activity.

**Immunoblotting.** Cell lysates from transfected HEK293T and COS7 cells and SF9 membrane expressing UGT1A5 protein (20–100 μg) were separated by 10% SDS–polyacrylamide gel electrophoresis and rectilinearly transferred to nitrocellulose (0.45 μm; Bio-Rad Laboratories, Hercules, CA). Immunodetection of UGT1A5 protein was performed using the WB-Human UGT1A1 Western Blotting Kit (BD Gentest, Woburn, MA). Nitrocellulose membranes were incubated with rabbit anti-UGT1A1 subclass IgG as the primary antibody (1:1500 dilution) followed by horseradish peroxidase–conjugated goat anti-rabbit IgG (Thermo Scientific, Rockland, IL) as the secondary antibody (1:2000 dilution). Additionally, SF9-expressed UGT1A5 was probed with His-tag–recognizing primary polyclonal antisera (rabbit-anti-human His-tagged UGT2B7) developed in this laboratory (Kerdpin et al., 2009). This antibody was raised to residues 55–165 of UGT2B7 and expressed in E. coli with a 6-histidine tag at the C terminus, and hence recognized His-tagged proteins. The primary antisera (1:1500 dilution) was detected using horseradish peroxidase–conjugated goat anti-rabbit IgG (1: 2000 dilution). Immunoreactivity was detected by chemiluminescence (Roche Diagnostics GmbH, Mannheim, Germany). Blots were visualized with a Fujifilm LAS-4000 imaging system (Fujifilm Life Sciences, NSW, Australia), and band intensities were measured using Multi Gauge software (Fujifilm Life Sciences). Relative UGT1A5 protein levels represent the mean of triplicate measurements. Western blot analysis and activity assays were performed using the same batch of cell lysate.

**Data Analysis.** Activity and kinetic data from experiments using uninfected SF9 membranes and c-SUP represent the mean of duplicate measurements unless otherwise indicated. For generation of kinetic constants, the Michaelis-Menten, Hill, and substrate inhibition equations were fit to untransformed experimental data using Enzfitter (Biosoft, Cambridge, UK) to generate kinetic parameters. Goodness of fit was assessed from the coefficient of determination (r²), F-statistic, 95% confidence intervals, and S.E. of the fit. Duplicate data were pooled for model fitting. Kinetic data are shown as Eadie-Hofstee plots (velocity vs. substrate [substrate]), and kinetic constants are reported as the parameter ± S.E. of the parameter fit.

**Michaels-Menten equation.**

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

where v is the rate of metabolite formation, V_max is the maximum velocity (as pmol/min-mg microsomal or cell lysate protein), [S] is the substrate concentration, and K_m is the Michaelis constant (substrate concentration at 0.5 V_max).

**Hill equation.**

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

where K_m is the Hill constant (substrate concentration at 0.5 V_max), and n is the Hill coefficient (n < 1 = negative cooperativity and n > 1 = positive cooperativity).

**Substrate inhibition.**

\[ v = \frac{V_{max}}{1 + \frac{S}{K_i} + \frac{S}{K_{m}}} \]

where K_i is the substrate inhibition constant.

Intrinsic clearance (Cl_intrinsic) was calculated as V_max/K_m.

**Results**

**Opioid Glucosidation.** We have reported previously that both c-SUP and Supersomes expressing UGT2B7 catalyze the 3-glucosidation, but not the 6-glucosidation, of MOR in the presence of UDP-Glc as cofactor (Chau et al., 2014). Thus, the kinetics of MOR 3-glucosidation by c-SUP and SF9 membranes were characterized using 11 substrate concentrations from 0.05 to 10 mM. Kinetic parameters are given as the mean of duplicate measurements ± S.E. of the parameter fit. MOR 3-glucosidation by c-SUP exhibited hyperbolic (Michaelis-Menten) kinetics, whereas negative cooperative kinetics (n = 0.96 ± 0.01) was observed with SF9 membranes (Fig. 1). Mean K_m (or S_50) values for MOR 3-glucosidation by c-SUP and SF9 membranes were 3.4 ± 0.001 and 4.4 ± 0.07 mM, respectively, and mean V_max values were 266 ± 9.3 and 362 ± 2.5 pmol/min-mg, respectively. In contrast to MOR, which has both phenolic (3-position) and enolic (6-position) hydroxyl groups, COD has only an enolic hydroxyl group at the 6-position. Consistent with the lack of MOR 6-glucosidation by c-SUP and SF9 membranes, COD was not glucosidated by these enzyme sources.

**Activity of Uninfected SF9 Membranes and c-SUP toward Hydroxyl-, Carboxylic Acid- and Amine-Containing Aglycones.** Screening studies were performed to further characterize the glucosidation capacity and selectivity of SF9 membranes and c-SUP. Twelve substrates that contained either an aliphatic or aromatic (phenolic) phenolic hydroxyl group, or a carboxylic acid or amine functional group were investigated. The activity profile of each substrate was determined at four concentrations that provided a meaningful activity range while maintaining aglycone solubility in the incubation medium.

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Glucosidation of Substrates Containing a Hydroxyl Group. In addition to MOR and COD, an additional eight compounds containing a phenolic or aliphatic hydroxyl group were screened for glucosidation by c-SUP and S9 membranes with UDP-Glc as cofactor (Fig. 2): 21-OHPr, 1-OHP, 4-MU, MPA, 1-NAP, 4-NP, PE, and AZT. AZT was glucosidated only by c-SUP (Fig. 2H). The rates of glucosidation of 21-OHPr, 1-OHP, and 1-NAP were higher with c-SUP compared with S9 membranes (Fig. 2, A, B, and E). At the highest aglycone concentration investigated, rates of glucosidation were approximately 22-, 28-, and 2.7-fold higher for 21-OHPr, 1-OHP, and 1-NAP, respectively. By contrast, the rates of formation of the glucosides of PE and MPA (phenolic) by S9 membranes were approximately 4- to 10.5-fold higher and 3- to 6-fold higher, respectively, compared with c-SUP (Fig. 2, D and H). The rates of 4-MU and 4-NP glucosidation were reasonably similar with both enzyme sources (Fig. 2, C and F).

To further characterize the glucosidation of hydroxyl-containing substrates, the kinetics of 1-OHP (Fig. 3, A and B), MPA (Fig. 3, C and D), and 4-MU (Fig. 3, E and F), glucosidation by c-SUP and S9 membranes were investigated using 11 or 12 aglycone concentrations that spanned the Km (or S50). Substrate concentration ranges are given in the Fig. 3 caption. Best-fit kinetic equations were consistent with the activity data shown in Fig. 2, and as observed with MOR, the equation of best fit differed between the two enzyme sources for 1-OHP and MPA. 1-OHP glucosidation by c-SUP exhibited negative cooperative kinetics (n = 0.89 ± 0.01) but weak substrate inhibition (Ksi = 13.3 ± 1.9 μM) with S9 membranes. Mean Km or S50 and Vmax values for 1-OHP glucosidation by c-SUP and S9 membranes were 8.0 ± 0.21 and 1.4 ± 0.11 μM and 11,211 ± 144 and 2713 ± 132 pmol/min/mg, respectively. MPA phenolic glucosidation by c-SUP and S9 membranes was best described by...
the Michaelis-Menten and substrate inhibition equations, respectively; mean $K_m$ and $V_{max}$ values for MPA phenolic glucosidation by c-SUP and Sf9 membranes were 165 $\pm$ 0.35 and 15.5 $\pm$ 1.1 $\mu$M ($K_{si}$ = 2998 $\pm$ 468 $\mu$M), and 916 $\pm$ 0.81 and 4076 $\pm$ 97 pmol/min/mg, respectively. 4-MU glucosidation by both c-SUP and Sf9 membranes exhibited negative cooperative kinetics with mean $n$, $S_{50}$, and $V_{max}$ values of 0.85 $\pm$ 0.003, 282 $\pm$ 2.6 $\mu$M, and 2390 $\pm$ 9.3 pmol/min/mg, respectively, for c-SUP, and 0.91 $\pm$ 0.03, 123 $\pm$ 8.3 $\mu$M, and 2580 $\pm$ 63 pmol/min/mg, respectively, for Sf9 membranes.

Glucosidation of Carboxylic Acid- and Amine-Containing Substrates. Rates of the acyl glucosidation of MPA and S-NAP, and the $N$-glucosidation of the amines BZC, LTG, and TFP by c-SUP and Sf9 membranes are shown in Fig. 2. Rates of S-NAP glucosidation were substantially higher (3- to 16-fold) with Sf9 membranes than with c-SUP (Fig. 2J). MPA acyl glucosidation was observed only with Sf9 membranes at the highest aglycone concentration (Fig. 2I). Similarly, LTG and TFP were glucosidated solely by Sf9 membranes (Fig. 2, L and M), and rates of BZC $N$-glucosidation by Sf9 membranes were more than double those of c-SUP (Fig. 2K).

Verification of Glucoside Formation by c-SUP and Uninfected Sf9 Membranes

Peaks corresponding to glucoside conjugates were not observed in chromatograms from experiments performed in the absence of UDP-Glc. As noted in Methods, authentic glucoside conjugates were available for BZC, 21-OHPr, MPA, 4-MU, and 4-NP. Glucosidation of these compounds was confirmed by comparison of HPLC retention times with those of authentic standards. In addition, the formation of a glucoside conjugate of the substrates investigated here was confirmed by LC-MS. Observed and predicted m/z values for glucosides, except those of 4-NP, are shown in Table 1. In addition, fragmentation patterns were consistent with glucoside formation (data not shown). An m/z value corresponding to 4-NP glucoside could not be detected by MS in positive ion mode.
even for the authentic standard, despite detection by HPLC and UPLC. However, the fragmentation pattern was consistent with formation of 4-NP glucoside.

Expression and Activity of UGT1A5 in Mammalian (HEK293T and COS7) and Insect (Sf9) Cell Lines

Initial experiments sought to express human UGT1A5 in HEK293T and COS7 cells. Expression of UGT1A5 protein was not apparent in HEK293T cells (Fig. 4A), but expression was observed in COS7 cell lysate (Fig. 4B). Although 1-OHP has been reported to be glucuronidated by UGT1A5 (Finel et al., 2005), glucuronidation of this substrate was not observed with either the transfected HEK293T or COS7 cell lysates. 1-OHP glucuronidation was confirmed with human liver microsomes as the positive control (data not shown).

Given the lack of or relatively weak expression of UGT1A5 in the mammalian cell lines and the lack of observed 1-OHP glucuronidation activity, baculovirus-mediated expression of His-tagged UGT1A5 in Sf9 cells was undertaken. Western blot analysis using an antibody that recognizes His-tagged proteins identified a band with the expected molecular mass of UGT1A5 (Fig. 4C). As with the mammalian expression systems, the UGT1A5 protein expressed in Sf9 cells lacked glucuronidation activity toward 1-OHP. However, incubations of the enriched membrane fraction of Sf9 cells expressing UGT1A5 and uninfected Sf9 cells with UDP-GlcUA as the added cofactor showed the presence of a peak that chromatographed with almost the same retention time as 1-OHP glucuronide. The second peak was identified as 1-OHP glucoside by LC-MS and by comparison of the HPLC retention time with that of the authentic standard. Incubation of uninfected Sf9 cell membranes supplemented with UDP-Glc as cofactor resulted in the formation of a 1-OHP glucoside peak that had an approximate 800-fold greater area than the peak formed with UDP-GlcUA as cofactor. The 1-OHP glucoside peak that formed with UDP-GlcUA as cofactor was presumed to arise from the presence of UDP-Glc as an impurity in commercial UDP-GlcUA. HEK293T, COS7, and Sf9 cells engineered to express recombinant UGT1A5 were additionally screened for 1-NAP, 4-MU, TFP, and LTG glucuronidation, but no activity was observed. UGT1A6 and UGT1A4 expressed in HEK293T cells were used as positive controls for the glucuronidation of 4-MU/1-NAP and TFP/LTG, respectively, as described by Uchaipichat et al. (2006) and Kubota et al. (2007).

Discussion

There is increasing evidence demonstrating that several human UGT1A and 2B subfamily enzymes may catalyze both glucuronidation and glucosidation reactions. Indeed, the importance of glucosidation as a drug and chemical biotransformation pathway may be underestimated (see Introduction). Recombinant UGT enzymes are used extensively for the reaction phenotyping of drug glucuronidation (Miners et al., 2010; Zientek and Youdim, 2015) and have also been used to investigate glucosidation (e.g., Mackenzie et al., 2003; Tang et al., 2003; Toide et al., 2004; Buchheit et al., 2011; Chau et al., 2014). Numerous mammalian and nonmammalian expression systems are used for the generation of recombinant UGT proteins, including baculovirus-infected insect (Sf9 and T. ni) cells (Radominska-Pandya et al., 2005). Recombinant human UGT enzymes expressed in insect cells are

### Table 1

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<th>Xenobiotic</th>
<th>Predicted Glucoside</th>
<th>Sf9 Membranes m/z</th>
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<tr>
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<td>448.18</td>
<td>448.18</td>
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<tr>
<td>Mycophenolic acid (phenolic and acyl)</td>
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<tr>
<td>1-Naphthol</td>
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<td>307.13</td>
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<tr>
<td>S-Naproxen</td>
<td>410.18*</td>
<td>410.16*</td>
<td>410.18*</td>
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<tr>
<td>S-Naproxen + Na adduct</td>
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<td>415.13*</td>
<td>415.11*</td>
<td></td>
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<tr>
<td>S-Naproxen + K adduct</td>
<td>431.11*</td>
<td>431.11*</td>
<td>431.10*</td>
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<tr>
<td>Trifluoperazine</td>
<td>571.23</td>
<td>571.22</td>
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<tr>
<td>Zidovudine</td>
<td>430.16</td>
<td>ND</td>
<td>431.16</td>
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</tbody>
</table>

ND, not detected.

* S-naproxen + NH₄ adduct.
* S-naproxen + Na adduct.
* S-naproxen + K adduct.

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Fig. 4. Immunoblots of UGT1A5 expressed in HEK293T (Panel A), COS7 (Panel B) and Sf9 (Panel C) cells. Lane 1, wild-type UGT1A5; Lane 2, UGT1A5-His40Pro; Lane 3, UGT1A5-Thr36Ile; Lane 4, positive controls (UGT1A1 (panels A and B) and His-tagged CYP1A1 (panel C)); Lane 5, negative controls (untransfected HEK293T (panel A) and COS7 (panel B) cell lysate, and uninfected Sf9 (panel C) cell membranes). Immuno-reactive bands are observed at 55 kDa for UGT1A5 and its mutants, 58 kDa for CYP1A1 and 60 kDa for UGT1A1. Western blots were performed in duplicate.
Drug and Chemical Glucosidation by Insect Cell Lines

By way of comparison, the Km/S50 values for MOR 3-glucosidation by c-SUP (0.95), and 4-MU (0.40) but considerably lower for MPA (0.02). Ratios (c-SUP/Sf9 membranes) were of a similar order for 1-OHP (0.73), were close to 1 for substrates exhibiting negative cooperative kinetics), c-SUP nor Sf9 membranes catalyzed the 6-glucosidation of COD and glucosyltransferases of c-SUP and Sf9 membranes. Although neither xenobiotics. However, differences occur between the native UDP-glucosyltransferase(s) capable of glucosidating drugs and other chemicals. This prompted us to investigate the glucosidation of a series of aglycones with a phenolic (1-OHP, 4-MU, MPA, 1-NAP, and 4-NP), aliphatic alcohol (COD, 21-OHP, PE, and AZT), acyl (MPA and S-NAP), or amine (BZC, LTG, and TFP) acceptor functional group (see Supplemental Fig. 1 for structures) by c-SUP and S9 membranes to characterize the scope and selectivity of drug and chemical glucosidation by these insect cell lines. All of the compounds investigated are known to be glucuronidated by human liver microsomes and/or recombinant UGTs (Green and Tephly, 1996; Shipkova et al., 2001; Stone et al., 2003; Uchaipichat et al., 2004, 2006; Finel et al., 2005; Rowland et al., 2006; Bowalgaha et al., 2007; Gaganis et al., 2007; Kabota et al., 2007; Raungrut et al., 2010).

Differences were observed in the substrate selectivities and activities of the native UDP-glucosyltransferases of c-SUP and S9 membranes. Among the phenols, rates of 1-OHP and 1-NAP glucosidation were substantially higher with c-SUP, while MPA was preferentially glucosidated by S9 membranes. Rates of glucosidation of 4-MU and 4-NP were similar with both enzyme sources. The aliphatic alcohols AZT and 21-OHP were solely or preferentially glucosidated by c-SUP, while rates of PE glucosidation were higher with S9 membranes. Neither c-SUP nor S9 membranes glucosidated MOR and COD at the 6-position (enolic). S9 membranes glucosidated the carboxylic acid functional group of MPA and S-NAP and N-glucosidated BZC, LTG, and TFP. By contrast, glucosidation activity of c-SUP was not measurable (MPA, LTG, and TFP) or was low in comparison with S9 membranes (S-NAP and BZC).

Differences in the kinetics of 1-OHP, MPA, and MOR (3-position) glucosidation were also observed between the two enzyme sources: 1-OHP, negative cooperative (c-SUP) and substrate inhibition (S9); MPA, Michaelis-Menten (c-SUP) and substrate inhibition (S9); and MOR, Michaelis-Menten (c-SUP) and substrate inhibition (S9). By contrast, 4-MU glucosidation by both enzyme sources exhibited negative cooperative kinetics. When data are considered as intrinsic clearances (calculated as \( \frac{K_m}{V_{max}} \), noted that \( n \) values were close to 1 for substrates exhibiting negative cooperative kinetics), ratios (c-SUP/S9 membranes) were of a similar order for 1-OHP (0.73), MOR (0.95), and 4-MU (0.40) but considerably lower for MPA (0.02). By way of comparison, the \( \frac{K_m}{V_{max}} \) values for MOR 3-glucosidation by c-SUP and S9 membranes (3.42–4.40 mM) were similar to the \( K_m \) (5.56 mM) reported for MOR 3-glucosidation by human liver microsomes, although the \( V_{max} \) was lower (Chau et al., 2014). Notably, 1-OHP was glucosidated very efficiently by c-SUP and S9 membranes, with \( CL_{int} \) values of 1409 and 1938 \( \mu l/mg \) min, respectively.

Taken together, the results demonstrate that c-SUP and S9 membranes have the capacity to glucosidate both drugs and nondonor xenobiotics. However, differences occur between the native UDP-glucosyltransferases of c-SUP and S9 membranes. Although neither c-SUP nor S9 membranes catalyzed the 6-glucosidation of COD and MOR and \( CL_{int} \) ratios were similar for several phenols (1-OHP, MOR, and 4-MU), S9 membranes preferentially glucosidated MPA while the aliphatic alcohols 21-OHP and AZT were glucosidated almost exclusively by c-SUP. S9 membranes exclusively or preferentially glucosidated the carboxylic acid- and amine-containing aglycones investigated here.

While it is acknowledged that too few compounds were studied to establish meaningful structure-function relationships, it is apparent that care is required when investigating drug and chemical glucosidation by recombinant UGT enzymes expressed in insect cells. As noted previously, there is evidence demonstrating that UGT-catalyzed glucuronidation and glucosidation may occur as complementary metabolic pathways for xenobiotics. For example, we observed MOR 3-glucosidation by Supersomes expressing UGT2B4, UGT2B7, UGT2B15, and UGT2B17, but activity was only apparent for UGT2B7 when the background activity of c-SUP was taken into account (Chau et al., 2014). By contrast, HEK293 cells do not express an endogenous UDP-glycosyltransferase capable of glucosidating MOR and other xenobiotics (Chau et al., 2014). The data emphasize the requirement for “control” cell lysate/membranes in the investigation of drug and chemical glucosidation (and possibly conjugation with other sugars) by recombinant enzymes expressed in insect cells. It is known that many insect species, including lepidopterans (which include \( S. frugiperda \) and \( T. ni \)), express UDP-glycosyltransferases that preferentially use UDP-Glc as cofactor for the metabolism of dietary and environmental chemicals (Ahn et al., 2012; Meech et al., 2012). It has also been proposed that insect viruses have evolved UDP-glycosyltransferases that apparently facilitate exploitation of insect larvae as hosts for reproduction (Meech et al., 2012), although it is unknown whether the viral vector (AcMNPV) used here expresses a xenobiotic UDP-glucosyltransferase.

Coincident with the study investigating xenobiotic glucosidation by c-SUP and uninfected S9 membranes, we commenced an investigation of UGT1A5 structure function. UGT1A5 expressed in S9 cells has been reported to readily glucuronidate 1-OHP (Finel et al., 2005). Using UDP-GlcUA as cofactor, the rate of 1-OHP glucuronidation (at a substrate concentration of 500 \( \mu M \)) by UGT1A5 was 97 pmol/min-mg. By contrast, rates of glucuronidation of 4-MU and scopoletin were low, approximately 1 pmol/min-mg. In the present work, expression of UGT1A5 was observed in COS7 cells, but expression was not apparent in HEK293T cells using a commercial UGT1A1 subfamily antibody. Moreover, 1-OHP glucuronidation was not observed with lysates of COS7 and HEK293T cells, despite being readily measurable with human liver microsomes as the enzyme source (approximately 5000 pmol/min-mg at ASPET Journals on September 17, 2023 dmd.aspetjournals.org Downloaded from 2005; Rowland et al., 2006; Bowalgaha et al., 2007; Gaganis et al., 2007; Kabota et al., 2007; Raungrut et al., 2010).

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from the presence of UDP-Glc as an impurity in UDP-GlcUA. Nevertheless, identification of the glycoside conjugate(s) formed by incubations of insect cell membranes with UDP-GlcUA is recommended, especially when the rate of product formation is low. In addition to Finel et al. (2005) and the work presented here, UGT1A5 expressed in COS7 cells has been reported to glucuronidate 7-ethyl-10-hydroxy-campothecin (SN-38), but the rate of glucuronidation was extremely low (ca. 100 pmol/16 h·mg, equivalent to 0.1 pmol/min·mg), and a nonspecific radiometric thin layer chromatographic method was used for product quantification (Ciotti et al., 1999). More recently, Yang et al. (2018) described the expression of active UGT1A5 and two polymorphic variants (UGT1A5*8 and UGT1A5*9) in fusion yeast (Schizosaccharomyces pombe) cells. The activities of Triton X-100 permeabilized cells expressing the UGT1A5 enzymes were investigated using the UGT-Glo assay (Promega, Madison, WI), which measures the depletion of proluciferin substrates (UGT-Glo substrates A and B) rather than metabolite formation. Moreover, the activity (or lack thereof) of control (untransformed) fusion yeast cells was not reported. Further studies are required to unambiguously characterize the function role of UGT1A5.

Acknowledgments

Technical support from David Elliot is acknowledged. We are grateful to Dr. Andrew Rowland for his helpful advice.

Authorship Contributions

Participated in research design: Chau, Lewis, Mackenzie, Miners.

Conducted experiments: Chau, Kaya.

Performed data analysis: Chau, Lewis, Miners.

Wrote or contributed to the writing of the paper: Chau, Lewis, Mackenzie, Miners.

References


Address correspondence to: John O. Miners, Department of Clinical Pharmacology, College of Medicine and Public Health, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia. E-mail: john.miners@flinders.edu.au