Morphine Glucuronidation and Glucosidation Represent Complementary Metabolic Pathways That Are Both Catalyzed by UDP-Glucuronosyltransferase 2B7: Kinetic, Inhibition, and Molecular Modeling Studies

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ABSTRACT

Morphine 3-β-D-glucuronide (M3G) and morphine 6-β-D-glucuronide (M6G) are the major metabolites of morphine in humans. More recently, morphine-3-β-D-glucoside (M-3-glucoside) was identified in the urine of patients treated with morphine. Kinetic and inhibition studies using human liver microsomes (HLM) and recombinant UGTs as enzyme sources along with molecular modeling were used here to characterize the relationship between morphine glucuronidation and glucosidation. The M3G to M6G intrinsic clearance (CLint) ratio (~5.5) from HLM supplemented with UDP-glucuronic acid (UDP-GlcUA) alone was consistent with the relative formation of these metabolites in humans. The mean CLint values observed for M-3-glucoside by incubations of HLM with UDP-glucose (UDP-Glc) as cofactor were approximately twice those for M6G formation. However, although the M3G-to-M6G CLint ratio remained close to 5.5 when human liver microsomal kinetic studies were performed in the presence of a 1:1 mixture of cofactors, the mean CLint value for M-3-glucoside formation was less than that of M6G. Studies with UGT enzyme-selective inhibitors and recombinant UGT enzymes, along with effects of BSA on morphine glycosidation kinetics, were consistent with a major role of UGT2B7 in both morphine glucuronidation and glucosidation. Molecular modeling identified key amino acids involved in the binding of UDP-GlcUA and UDP-Glc to UGT2B7. Mutagenesis of these residues abolished morphine glucuronidation and glucosidation. Overall, the data indicate that morphine glucuronidation and glucosidation occur as complementary metabolic pathways catalyzed by a common enzyme (UGT2B7). Glucuronidation is the dominant metabolic pathway because the binding affinity of UDP-GlcUA to UGT2B7 is higher than that of UDP-Glc.

Introduction

Morphine remains the most widely prescribed analgesic for the treatment of moderate to severe pain and is also used as an opioid adjunct in general anesthesia, for the relief of severe dyspnea, and as an adjunct for acute pulmonary edema. It is generally accepted that glucuronidation at the 3- and 6-positions to form morphine-3-β-D-glucuronide (M3G) and morphine-6-β-D-glucuronide (M6G), respectively, are the major metabolic pathways of morphine in humans. M3G, M6G, and unchanged drug account on average for 55, 10, and 8% of the urinary recovery, respectively, of an intravenous dose of morphine in humans along with trace amounts of normorphine, morphine-3-sulfate, and morphine-3,6-diglucuronide (Hasselström and Säwe, 1993; Milne et al., 1996). More recently, however, Chen et al. (2003) identified morphine-3-β-D-glucoside (M-3-glucoside) and morphine-6-β-D-glucoside (M-6-glucoside) in the urine of cancer patients treated with morphine, although the 6-glucoside was excreted in trace amounts only. Thus, M-3-glucoside may contribute to the deficit in metabolite recovery observed in earlier studies. It is unknown whether the morphine glucosides are pharmacologically active, as are M3G and M6G (Frances et al., 1992; Milne et al., 1996).

Drug and chemical glucosidation is a poorly characterized metabolic pathway compared with glucuronidation. However, there is evidence demonstrating that a number of drugs and other compounds cleared by glucuronidation also undergo glucosidation (Meech et al., 2012). Both glucuronidation and glucosidation have been reported for AS-3201 (R-(−)-2-(4-bromo-2-fluorobenzyl)-1,2,3,4-tetrahydropyrrolo[1,2-a]pyrazine-4-spiro-3′-pyrrolidine-1′,2′,3,5′-tetrone; an aldose reductase inhibitor), bilirubin, hydoxycholyl acid, ibuprofen, mycophe- nolic acid, varenicline, and an experimental endothelin ETA

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ABBREVIATIONS: AS-3201, R-(−)-2-(4-bromo-2-fluorobenzyl)-1,2,3,4-tetrahydropyrrolo[1,2-a]pyrazine-4-spiro-3′-pyrrolidine-1′,2′,3,5′-tetrone; AZT, zidovudine; BSA, bovine serum albumin; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; 4-MU, 4-methylumbelliferone; M3G, morphine-3-β-D-glucuronide; M6G, morphine-6-β-D-glucuronide; M-3-glucoside, morphine-3-β-D-glucoside; UDP-Glc, UDP-glucose; UDP-GlcUA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.
receptor antagonist (Toide et al., 2004; Fewer et al., 1977; Shipkova et al., 1999, 2001; Mackenzie et al., 2003; Tang et al., 2003; Obach et al., 2006; Buchheit et al., 2011). UDP-glucose (UDP-Glc) and UDP-glucuronic acid (UDP-GlcUA) are the respective cofactors for glucosidation and glucuronidation reactions. UDP-GlcUA is synthesized from UDP-Glc by a two-stage NAD+ -dependent reaction catalyzed by UDP-glucose dehydrogenase. Thus, both cofactors are available in mammalian cells for drug and chemical conjugation. Estimates of the ratio of UDP-GlcUA to UDP-Glc present in rat hepatocytes range from approximately 0.6 to 2 (Aw and Jones, 1982; Alary et al., 1992; Linster and Van Schaftingen, 2003).

Glucuronidation reactions are catalyzed by enzymes of the UDP-glucuronosyltransferase (UGT) superfamily. Nineteen human UGTs in the UGT1A, UGT2A, and UGT2B subfamilies that use UDP-GlcUA as cofactor have been identified to date (Miners et al., 2004; Mackenzie et al., 2005). The majority of these enzymes are expressed in liver, although hepatic expression of UGT 1A5, 1A7, 1A8, 1A10, 2B11, and 2B22 appears to be negligible. UDP-GlcUA binds to a highly conserved region in the C-terminal half of UGT proteins, with well-defined UDP and sugar binding domains (Miley et al., 2007; Radominska-Pandya et al., 2010). Although glucuronidation is the primary reaction catalyzed by UGT1 and UGT2 family enzymes, a number of UGTs may additionally use UDP-Glc as cofactor to form glucoside conjugates (Senafi et al., 1994; Mackenzie et al., 2003; Tang et al., 2003; Toide et al., 2004; Obach et al., 2006; Buchheit et al., 2011). In contrast, UGT3A1 and UGT3A2 use sugar donors other than UDP-GlcUA (Mackenzie et al., 2008, 2011). Notably, UGT3A2 glucosidates a broad range of xenobiotics and estrogens.

It is well established that UGT2B7 is primarily responsible for morphine 3- and 6-glucuronidation (Coffman et al., 1997; Stone et al., 2003; Ohno et al., 2008), although several UGT1A enzymes may also catalyze M3G formation. However, morphine glucosidation has not been investigated to date, despite the ongoing widespread use of this drug. Here we report kinetic and inhibition studies using human liver microsomes (HLM) and recombinant UGTs as the enzyme sources that sought to characterize the relationship between morphine glucuronidation and glucosidation. Kinetic studies were undertaken with morphine and cofactor (UDP-GlcUA and UDP-Glc) as the variable substrate in the presence and absence of bovine serum albumin (BSA). BSA (2% w/v) is known to enhance the activity of human liver microsomal and recombinant UGT2B7 and several other UGT enzymes by sequestration of inhibitory long-chain unsaturated fatty acids released from membranes during the course of an incubation, thereby providing more reliable estimates of enzyme kinetic parameters (Rowland et al., 2007, 2008; Kiflford et al., 2009; Manevski et al., 2011; Walsky et al., 2012). The kinetic and inhibitor studies were complemented by molecular modeling and site-directed mutagenesis to identify residues of UGT2B7 that bind UDP-GlcUA and UDP-Glc. The results provide a kinetic, enzymatic, and molecular basis for the interpretation of drug glycosidation and further demonstrate that glucuronidation and glucosidation may occur as complementary, parallel metabolic pathways.

Materials and Methods

Acetobromo-a-D-glucose (95%), BSA, M3G, M6G, alamethicin (from Trichoderma viride), heocogenin, lithium hydroxide monohydrate, niflumic acid, 1-octanesulfonic acid sodium salt, UDP-Glc (disodium salt), UDP-GlcUA (trisodium salt), and zidovudine (AZT) were purchased from Sigma-Aldrich (Sydney, Australia); morphine hydrochloride was from GlaxoSmithKline (Melbourne, Australia); and Supersomes expressing UGT2B4, UGT2B7, UGT2B15, and UGT2B17 and microsomes from untransfected Hi5 cells were from BD Biosciences (North Ryde, Australia). Fluconazole was provided by Pfizer Australia (Sydney, Australia). Solvents and other reagents, including acetic acid, acetone, methanol, and perchloric acid, were of analytical reagent grade.

Human Liver Microsomes and Expression of Recombinant UGTs. HLM were prepared from five human livers (three females, two males) obtained from the human liver bank of the Department of Clinical Pharmacology of Flinders University by differential centrifugation according to Bowellaha et al. (2005). Microsomes were preincubated with alamethicin (50 μg/mg protein) prior to incubation (Boase and Miners, 2002). Pooled HLM were prepared by mixing equal protein amounts from the five livers. The use of human liver tissue in xenobiotic metabolism studies was approved by the Flinders Clinical Research Ethics Committee.

UGT cDNAs were stably expressed in human embryonic kidney (HEK) 293 cells, as described previously (Stone et al., 2003; Uchaipichat et al., 2004). Cells were harvested and washed in phosphate-buffered saline once they had grown to at least 80% confluence, lysed by sonication, and centrifuged at 12,000 × g for 1 minute at 4°C. The supernatant fractions were separated and stored in phosphate buffer (0.1 M, pH 7.4) at −80°C until use. Expression of all UGTs was demonstrated by immunoblotting with a commercial UGT1A antibody (BD Gentest, Woburn, MA) or a nonselective UGT antibody raised against purified mouse Ugt (Uchaipichat et al., 2004) and by measurement of enzyme activities. The nonselective substrate 4-methylumbelliferone (4-MU) was used to confirm the catalytic activity of the majority of the UGT enzymes expressed in HEK293 and insect cells (Supersomes), including UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, 2B15, and 2B17 according to the procedure of Lewis et al. (2007). UGT1A4 and UGT2B4 activities were measured using lamotrigine and codeine as the respective substrates (Rowland et al., 2006; Raungrut et al., 2010), given the low or absent 4-MU glucuronidation activities of these enzymes.

Morphine 3- and 6-Glycosidation Assay. The method used to measure the formation of M3G, M6G, and M-3-glucoside was a modification of the procedure for morphine 3- and 6-glucuronidation reported by Uchaipichat et al. (2011). In brief, incubations, in a total volume of 200 μl, contained phosphate buffer (0.1 M, pH 7.4), MgCl2 (4 mM), alamethicin-activated HLM (0.1 mg), morphine, and UDP-GlcUA and/or UDP-Glc (5 mM, unless specified otherwise), with or without BSA (2% w/v). After a 5-minute preincubation at 37°C in a shaking water bath, reactions were initiated by the addition of cofactor (UDP-Glc and/or UDP-GlcUA) and performed for 30 minutes. Reactions were terminated by the addition of perchloric acid (70% v/v; 2 μl, or 8 μl for incubations containing BSA) and cooling on ice. Samples were centrifuged (5000g for 10 minutes), and a 10-μl aliquot of the supernatant fraction was analyzed by high-performance liquid chromatography (HPLC). Rates of M-3-glucoside, M3G, and M6G formation were linear with respect to protein concentration and incubation time under these conditions.

Experiments using recombinant human UGTs were as described for HLM, except for altered protein concentration (0.4 mg of HEK293 cell lysate or 0.1 mg of Supersome protein) and incubation time (120 minutes). Reactions were terminated with 1 μl of perchloric acid and a 10-μl aliquot of the supernatant fraction was analyzed by HPLC. Initially, experiments with recombinant UGT2B2 enzymes were conducted with Supersomes expressing UGT2B4, UGT2B7, UGT2B15, and UGT2B17 because of the relatively low activity of these enzymes expressed in HEK293 cells. However, later experiments also used UGT2B2 proteins expressed in HEK293 cells after the observation that insect (Hi5) cells expressed a native glucosyltransferase (see Results).
Acetobromo-synthesized using a modification of the procedure of Berrang et al. (1975). Quantification of M3G, M6G, and M-3-glucoside were 0.1, 0.25, and 0.1 sensitivity, defined as five times background noise, for the quantification of M3G, M6G, and M-3-glucoside standard curves (five concentrations in the range 0.25 to 10 μM). Coefficients of variation for the slopes of the M3G, M6G, and M-3-glucoside standard curves (n = 16) were 2.4, 3.4, and 0.9%, respectively. Overall assay reproducibility, determined by measuring M3G, M6G, and M-3-glucoside formation at low (0.5 mM) and high (10 mM) morphine concentrations for nine separate incubations of the same batch of HLM, ranged from 0.5 to 6.3%. The lower limits of sensitivity, defined as five times background noise, for the quantification of M3G, M6G, and M-3-glucoside were 0.1, 0.25, and 0.1 μM, respectively.

Synthesis of Morphine-3-Glucoside. M-3-glucoside was synthesized using a modification of the procedure of Berrang et al. (1975). Acetobromo-D-glucose (308 mg, 0.75 mmol) was added to a solution of morphine hydrochloride (394 mg, 1.05 mmol) and lithium hydroxide monohydrate (82 mg, 1.96 mmol) in dry methanol (15 ml), and the reaction mixture was stirred at room temperature for 30 minutes. An aqueous solution of lithium hydroxide (1.67 M, 1.5 ml) was added dropwise to the stirred reaction mixture. The mixture was stirred for a further 30 minutes, during which time a white precipitate formed. The solid was collected by filtration, washed with ice-cold methanol (1 ml), and dried under vacuum. The crude solid was dissolved in a minimal volume of hot methanol-water (1:1). On cooling a white solid formed that was collected by filtration and dried under vacuum (82 mg, 1.96 mmol) in dry methanol (15 ml), and the M-3-glucoside was selectively eluted with acetonitrile-water (1:9). The solvent was evaporated under a stream of nitrogen. Trituration of the resulting solid in ice-cold methanol (0.5 ml) produced crystalline M-3-glucoside, which was collected by filtration and dried under vacuum (6 mg, purity 99% by HPLC). The structure of the synthetic M-3-glucoside was confirmed by nuclear magnetic resonance (Supplemental Table 1). Mass spectrometry (AQUITY QTOF Premier; Waters Corporation) in positive ion gave a molecular ion of m/z 448.46 amu (predicted 448.48 amu).

Quantification of Morphine Binding to HLM and BSA. Binding of morphine to HLM and 2% (w/v) BSA was measured by equilibrium dialysis according to the general method of McLaren et al. (2000). Binding measurements were performed using a Dionex equilibrium dialysis apparatus comprising Teflon dialysis cells (capacity of 1.2 ml per side) separated into two compartments with Sigma-Aldrich dialysis membrane (molecular mass cutoff 12 kDa). One side of the dialysis cell was loaded with 1 ml of morphine (0.1 to 10 mM) in phosphate buffer (0.1 M, pH 7.4). The other compartment was loaded with 1 ml of either a suspension of HLM (0.5 mg/ml) in phosphate buffer (0.1 M, pH 7.4) or HLM (0.5 mg/ml) plus BSA (2% w/v) 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 also performed with phosphate buffer or HLM (0.5 mg/ml) and BSA (2% w/v) on both sides of the dialysis cells at low and high concentrations of morphine to ensure that equilibrium was attained. A 200-μl aliquot was collected from each compartment, treated with ice-cold methanol containing 4% glacial acetic acid (200 μl), and cooled on ice. Samples were subsequently centrifuged at 4000 g for 10 minutes at 10°C, and an aliquot of the supernatant fraction (5 μl) was analyzed by HPLC.

Morphine present in dialysate samples was measured using the HPLC instrument and column as described for the morphine glycosides. Separation was achieved using an isocratic mobile phase comprising of acetonic acid (5%) and glacial acetic acid (1%) in distilled water at a flow rate of 1 ml/min. The retention time for morphine under these conditions was 2.8 minutes. Morphine concentrations in dialysis samples were determined by comparison of peak areas to those of an authentic morphine standard curve prepared over the concentration range 0.1 to 10 mM. The lower limit of quantitation (assessed arbitrarily at 5 times the limit of detection) for morphine was 0.15 μM.

Inhibition of Human Liver Microsomal M-3-Glucoside Formation by UGT Enzyme Selective Substrates/Inhibitors and Cofactor Competition Studies. The effects of UGT enzyme selective inhibitors and/or substrates on M-3-glucoside formation with HLM as the enzyme source were investigated to confirm the involvement of specific hepatic UGTs in M-3-glucoside formation. Compounds screened for inhibition included niflumic acid (5 and 100 μM), hecogenin (10 μM), fluconazole (2.5 mM), and AZT (5 mM), which are selective inhibitors or substrates of UGT1A1/1A9, 1A4, and 2B7/2B4, respectively (Court et al., 2003; Uchaipichat et al., 2006a,b; Miners et al., 2011). The morphine concentration used in these experiments was 4 mM (see Results).

Inhibition of M-3-glucoside formation by UDP-GlcUA (five concentrations in the range 0.2–1 mM) at each of three UDP-Glc concentrations (1, 1.5, and 2.5 mM) was investigated at the absence and presence of BSA at a fixed saturating morphine concentration (20 mM). Additionally, inhibition of M3G and M6G formation by UDP-Glc (five concentrations in the range 1–3 mM) was investigated at each of three UDP-GlcUA concentrations (0.25, 0.5, and 1 mM) in the absence and presence of BSA at a fixed, saturating morphine concentration (20 mM).

Computational Modeling. The UGT2B7 homology model used in the present study was generated by Lewis et al. (2011) using FUGUE and ORCHESTRAR (SYBYL-X 1.2; Tripos, St. Louis, MO). The X-ray crystal structures of grape UDP-glucose flavanoid 3-O-glucosyltransferase (2C1X, 2C1Z, and 2C9Z), barbel medic UDP-glucose flavonoid/triterpene glucosyltransferase UGT71G1 (2ACV and 2ACW), and the cofactor binding domain of UGT2B7 (206L) (Miley et al., 2007), obtained from the Brookhaven Protein Data Bank, were used as templates for model construction. Alignment of the templates with UGT2B7 provided a residue consensus of 50%. UDP-GlcUA and UDP-Glc were independently docked into the active site of the UGT2B7 homology model using SurfexDock (SYBYL-X 1.2; Tripos). The major species of UDP-GlcUA and UDP-Glc at pH 7.4 have charges of -3 and -2, respectively, as determined by Marvin View 5.3.8 (Chem Axon, Budapest, Hungary). Hence, the carboxylate ion of UDP-GlcUA was docked. Proton exchange for each experiment was optimized based on preliminary docking data obtained using the red grape UDP-glucose/flavanoid 3-O-glucosyltransferase (VvGT1) X-ray crystal structure (Protein Data Bank code 2C1Z) (Ofen et al., 2006), as this protein was crystallized with bound cofactor. Docked poses were evaluated based on an energy consensus score (C_conc) and the SurfexDock scoring function. Energetically
favored poses were assessed for hydrogen bonding interactions (ΔΔG ≈ 2.8 Å), and the distance between the anomeric carbon of the sugar moiety and His35, the proposed catalytic base involved in proton abstraction during conjugation of the aglycone with the UDP-sugar, was measured.

**PCR Site-Directed Mutagenesis and Expression of Mutant UGT2B7 Proteins.** Based on the results of the docking and previously published data (see Results), mutants were generated at positions 33 (Y33F and Y33L), 378 (N378A, N378L, N378H, and N378Q), 398 (D398A and D398L), 399 (Q399A and Q399L), and 402 (N402A, N402H, N402L, and N402) by site-directed mutagenesis using the wild-type UGT2B7 cDNA in pBluescript II SK(+) as the template. Oligonucleotide primers used for mutagenesis are shown in Supplemental Table 2. The PCR site-directed mutagenesis reactions, in a total volume of 50 µl, contained 100 ng of DNA template, 125 ng of forward and reverse oligonucleotide primer, 0.2 mM of each dNTP, DMSO (5%, v/v), and 2.5 U PfuUltra II Fusion HS DNA Polymerase. Reaction mixtures were prepared in PfuUltra II Hot Start reaction buffer. The PCR reaction was performed using a Robocycler Gradient 96 temperature cycler (Stratagene, La Jolla, CA). Cycling parameters were as follows: 3 minutes at 95°C, followed by 16 cycles of 1 minute at 95°C for denaturation, 1 minute at 52°C for primer annealing, and 10 minutes at 68°C for extension, with a final extension of 60 minutes at 68°C. PCR products were purified using the QIAQuick PCR Purification Kit. The PCR product was then digested with 20 U of DpnI (37°C for 60 minutes) to cleave the methylated template plasmid and heat inactivated at 80°C for 20 minutes. After transformation into DH5α cells and purification, mutant UGT2B7 coding sequences were ligated into the mammalian vector pEF-IRES-Puro 5 using the restriction enzymes Xhol and XbaI. All mutations were confirmed on both strands by DNA sequencing using a 3130xl Genetic Analyzer with BigDye Terminator v3.1 Chemistry (Applied Biosystems, Foster City, CA). The wild-type and mutant cDNAs were transiently expressed in HEK293T cells, using 1 × 10⁵ cells/ml (70% confluent) containing 24 µg of plasmid DNA, Opti-MEM (1.5 ml), and Lipofectamine 2000 (60 µl). Cells were harvested as described previously.

Cell lysate from HEK293T cells expressing wild-type and mutant UGT2B7 proteins (50 µg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. IgG (H+L) secondary HRP-conjugated IgG (ImmunoPure; Thermo Scientific, Waltham, MA) was added to incubations containing 2% (w/v) BSA, was incubated for 60 minutes) to cleave the methylated template plasmid and heat inactivated at 80°C for 20 minutes. After transformation into DH5α cells and purification, mutant UGT2B7 coding sequences were ligated into the mammalian vector pEF-IRES-Puro 5 using the restriction enzymes Xhol and XbaI. All mutations were confirmed on both strands by DNA sequencing using a 3130xl Genetic Analyzer with BigDye Terminator v3.1 Chemistry (Applied Biosystems, Foster City, CA). The wild-type and mutant cDNAs were transiently expressed in HEK293T cells, using 1 × 10⁵ cells/ml (70% confluent) containing 24 µg of plasmid DNA, Opti-MEM (1.5 ml), and Lipofectamine 2000 (60 µl). Cells were harvested as described previously.

**Results**

**Morphine Binding to HLM and BSA.** As noted in Introduction, addition of BSA enhances the activity of UGT2B7 and several other UGT enzymes. Thus, BSA (2% w/v) was added to incubations of HLM. This necessitated investigation of the binding of morphine to HLM and BSA. The binding of morphine to HLM and BSA was determined as the concentration of drug in the buffer compartment divided by the concentration of drug in the protein compartment from equilibrium dialysis experiments. Binding to both HLM and BLM with BSA (2% w/v) was negligible (<10%) across the morphine concentration range investigated (0.1–10 mM). Hence, there was no requirement for the correction of morphine concentrations because of binding to incubation constituents in the calculation of kinetic parameters.

**Kinetics of Morphine Glycosidation by HLM (±BSA) in the Presence of a Single (UDP-Glc or UDP-GlcUA) Cofactor with Morphine as the Variable Substrate.** Morphine glucuronidation kinetics were initially characterized at saturating cofactor concentration (5 mM UDP-Glc or UDP-GlcUA) in both the absence and presence of 2% (w/v) BSA. With UDP-Glc alone as cofactor, M-3-glucosidation exhibited Michaelis-Menten kinetics in all five livers in the absence and presence of BSA (Fig. 1A) for morphine concentrations in the range 0.1–10 mM. There was no evidence from HPLC, indicating the formation of a second morphine glucoside (i.e., M-6-glucoside). Derived kinetic constants (n = 5 livers) are shown in Table 1. The addition of BSA to incubations resulted in an 89% decrease (P < 0.01) in the mean Km (5.56 to 0.63 mM) for M-3-glucoside formation, with no significant change in Vmax. There was a corresponding 8.5-fold (P < 0.01) increase in the mean CLint (0.3 to 2.54 µl/min mg). The Km for M-3-glucoside formation, in both the presence and absence of BSA, was ~40 to 90% higher than the S50 (substrate concentration at 1/2 Vmax from the Hill equation) or Km values for morphine 3- and 6-glucuronidation, whereas Vmax and CLint values were lower and higher than the corresponding parameters for M3G and M6G formation, respectively (see below and Table 1).

By contrast, with UDP-GlcUA as cofactor M3G formation in the five livers investigated followed negative cooperative kinetics, both in the absence and presence of BSA (Fig. 1B). Morphine glucuronidation by HLM and UGT2B7 has previously been shown to exhibit atypical kinetics (Miners et al., 1988; Stone et al., 2003). Derived S50 and Vmax values from fitting with the Hill equation are shown in Table 1. Although it is strictly not valid to calculate CLint (as Vmax/S50) for negative cooperative kinetics, values of the Hill coefficient (n) approached unity (range 0.77 to 0.93; Table 1), and hence S50 and Vmax/S50 approximate Km and CLint, respectively. As observed for M-3-glucosidation, the addition of BSA to incubations resulted in an 88% decrease (P < 0.01) in mean S50 with a modest (13.3%, P < 0.05) decrease in mean Vmax. This resulted in a 6.7-fold (P < 0.01) increase in the mean...
CLint. The Hill coefficient did not change appreciably in the presence of BSA. Similar to M3G, M6G formation followed negative cooperative kinetics in the absence of BSA but exhibited weak substrate inhibition (Km..Ksi) in the presence of BSA (Fig. 1C and Table 1). The addition of BSA to the incubation resulted in an 88% decrease (P < 0.01) in mean S50 or Km, from 2.88 to 0.36 mM, with a nonsignificant decrease in Vmax. Mean CLint was a 6.8-fold higher (P < 0.01) in the presence of BSA.

Kinetics of Morphine Glycosidation by HLM (±BSA) in the Presence of Combined (UDP-Glc plus UDP-GlcUA) Cofactors with Morphine as the Variable Substrate. In the presence of UDP-Glc plus UDP-GlcUA, both 5 mM (±BSA, 2% w/v), there were statistically significant (P < 0.05) changes in all M-3-glucoside kinetic parameters compared with data generated in the presence of UDP-Glc alone (Table 1). Whereas M-3-glucoside formation kinetics exhibited negative cooperativity in the presence of both cofactors...

### TABLE 1

**Derived morphine glycosidation kinetic constants generated in the absence and presence of BSA (2% w/v) with morphine as the variable substrate**

Data are shown as the mean ± S.D. for HLM from five separate livers.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Cofactor</th>
<th>Without BSA</th>
<th>With BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Km or S50</td>
<td>Vmax</td>
</tr>
<tr>
<td>M6G</td>
<td>UDP-GlcUA</td>
<td>2.88 ± 1.21</td>
<td>357 ± 79</td>
</tr>
<tr>
<td>M3G</td>
<td>UDP-GlcUA</td>
<td>3.63 ± 1.11</td>
<td>2803 ± 599</td>
</tr>
<tr>
<td>M-3-glucoside</td>
<td>UDP-Glc</td>
<td>3.56 ± 1.03</td>
<td>1581 ± 631</td>
</tr>
</tbody>
</table>

### Notes:

- aP < 0.05; comparisons for each glycoside ± BSA for single cofactor experiments.
- bP < 0.01; comparisons for each glycoside ± BSA for single cofactor experiments.
- cP < 0.05; comparisons for each glycoside between single and mixed cofactor experiments.
- dP < 0.01; comparisons for each glycoside between single and mixed cofactor experiments.
- 1:1; combination of 5 mM UDP-Glc and 5 mM UDP-GlcUA.
- Kd for M6G formation in presence of BSA = 62 ± 6 mM.
plus BSA, Michaelis-Menten kinetics were observed in the absence of BSA (Fig. 1D). The combination of cofactors resulted in modest changes (~30–40% increase or decrease) in the mean $K_m$ or $S_{50}$ for M-3-glucoside formation but large decreases in $V_{\text{max}}$ (~85%) and intrinsic clearance (77 to 89%), both in the absence and presence of BSA. As observed in the presence of UDP-Glc alone, addition of BSA to incubations resulted in a significant decrease in mean $K_m$ (or $S_{50}$) for M-3-glucoside formation (by 77%) without an effect on $V_{\text{max}}$ in the presence of the mixed cofactors (Table 1).

Similar to M-3-glucoside formation, the addition of the second cofactor, in this case UDP-Glc, modestly altered the mean $K_m$ or $S_{50}$ values for M3G and M6G formation (35 to 64% increases), both in the absence and presence of BSA (Table 1; Fig. 1, E and F). However, in contrast to the 3-glucosidation pathway, changes in $V_{\text{max}}$ values for M3G and M6G in the presence of both cofactors were minor or negligible. As observed in the single cofactor experiments, BSA decreased the mean $K_m$ and $S_{50}$ values for M3G and M6G formation (by 88%) in the presence of the mixed cofactors, without significantly affecting $V_{\text{max}}$ (Table 1).

**Kinetics of Morphine Glycosidation by Pooled HLM (± BSA) with Cofactor as the Variable Substrate.** To validate the use of pooled HLM in subsequent cofactor kinetic and inhibition studies, the kinetics of morphine glucosidation and glucuronidation by a pool of the five separate livers (equal protein amounts) used in the morphine glycosidation studies described above were characterized. Kinetic models of M-3-glucoside, M3G, and M6G formation by pooled HLM were identical to those observed for the individual livers and kinetic parameters ($K_m$, $V_{\text{max}}$, $n$, and $K_i$) were within 20% of the mean values for the individual livers (data not shown).

UDP-Glc and UDP-GlcUA kinetics using pooled HLM were characterized separately at a fixed, saturating concentration of morphine (20 mM), with and without BSA. UDP-Glc and UDP-GlcUA kinetics exhibited negative cooperative kinetics over the cofactor concentration range 0.05–10 mM (Fig. 2, A–C). The $S_{50}$ values for UDP-GlcUA were similar with respect to both M3G and M6G formation (Table 2). However, the $S_{50}$ for UDP-Glc, measured with respect to M-3-glucoside formation, was approximately threefold higher than the values for UDP-GlcUA. Although the $S_{50}$ for UDP-Glc was unaffected by BSA, the addition of BSA to incubations resulted in a 40 to 60% reduction in the $S_{50}$ for UDP-GlcUA (Table 2; Fig. 2, A–C). Small to modest increases in the $V_{\text{max}}$ values for UDP-Glc and UDP-GlcUA (measured with respect to M3G formation) were additionally observed.

**Cofactor Inhibition Studies.** Dixon plots for inhibition of M-3-glucoside formation by UDP-GlcUA and inhibition of M3G and M6G formation by UDP-Glc in the absence and presence of BSA at a fixed, saturating morphine concentration (20 mM) are shown in Fig. 3. UDP-GlcUA competitively inhibited M-3-glucoside formation, with $K_i$ values of 0.15 ± 0.01 mM (parameter ± S.E.M. of parameter fit) and 0.18 ± 0.01 mM in the absence and presence of BSA, respectively (Fig. 3, A and B). Likewise, UDP-Glc was a competitive inhibitor of both M3G and M6G formation. $K_i$ values with respect to M3G were 2.27 ± 0.13 and 1.73 ± 0.27 mM in the absence and presence of BSA, respectively, whereas $K_i$ values with respect to M6G were of 2.49 ± 0.19 and 1.63 ± 0.25 mM in the absence and presence of BSA, respectively (Fig. 3, C–F).

**Morphine Glycoside Formation by Recombinant UGTs.** Recombinant UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, and 1A10 (expressed in HEK293 cells) and 2B4, 2B7, 2B15, and 2B17 (expressed in HEK293 cells and as Supersomes) were used in activity screening experiments to identify enzymes with the capacity to catalyze morphine glucuronidation and glucosidation. The activity screening experiments were conducted at three morphine concentrations: 1, 5, and 10 mM. Consistent with previous data from this laboratory using UGT enzymes expressed in HEK293 cells (Stone et al., 2003), UGT2B7 exhibited highest morphine 3-glucuronidation activity and only UGT2B7 formed M6G (Supplemental Table 3). Likewise, only UGT2B7 catalyzed M-3-glucosidation; rates of M-3-glucoside formation are shown in Supplemental Table 3. By contrast, Supersomes expressing UGT2B4, 2B7, 2B15, and 2B17 all formed M-3-glucoside (Fig. 4), although only UGT2B4 (minor) and UGT2B7 (major) formed M3G and M6G. Interestingly, however, untransfected Supersomes formed substantial amounts of M-3-glucoside (Fig. 4). When the endogenous glucosidation activity was taken into account, only UGT2B7 had significant remaining M-3-glucosidation activity.

**Inhibition of Human Liver Microsomal Morphine 3-Glucoside Formation by UGT Selective Inhibitors.** AZT, fluconazole, hecogenin, and niflumic acid were screened as inhibitors of morphine glucosidation and glucuronidation by pooled HLM. The morphine concentration used in the

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**Fig. 2.** Eadie-Hofstee plots for morphine glycosidation by pooled HLM (± BSA, 2% w/v) at a fixed morphine concentration (20 mM) with UDP-Glc and UDP-GlcUA as the variable substrates: (A) M-3-glucoside formation with UDP-Glc as cofactor; (B) M3G formation with UDP-GlcUA as cofactor; (C) M6G formation with UDP-GlcUA as cofactor. Points represent the mean values of quadruplicate measurements.
inhibition studies was 4 mM, which is between the mean $K_{m}/S_{50}$ values for M-3-glucoside (5.56 mM), M3G (3.63 mM), and M6G (2.88 mM) formation (Table 1). Fluconazole, a selective inhibitor of UGT2B7/2B4 (Uchaipichat et al., 2006b; Raungrut et al., 2010), and AZT, a selective substrate of UGT2B7 (Court et al., 2003), reduced M-3-glucoside, M3G, and M6G formation by 70–80, 50–65, and 55–65%, respectively (Fig. 5). The extent of inhibition of morphine glycosidation by fluconazole (2.5 mM) observed here is typical of the effect of this compound on glucuronidation reactions catalyzed by recombinant UGT2B7 (Uchaipichat et al., 2006b; Raungrut et al., 2010). Hecogenin, a selective inhibitor of UGT1A4 (Uchaipichat et al., 2006a), and niflumic acid, which inhibits UGT1A1, 1A9, and 2B15 at concentrations ranging from 5 to 100 μM (Miners et al., 2011), had no effect on morphine conjugate formation.

### TABLE 2

<table>
<thead>
<tr>
<th>Cofactor (Pathway)</th>
<th>Without BSA</th>
<th>With BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$S_{50}$ (mM)</td>
<td>$V_{max}$ (pmol/min/mg)</td>
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<tr>
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<tr>
<td>UDP-Glc (M-3-glucoside)</td>
<td>1.98 ± 0.19</td>
<td>1701 ± 48</td>
</tr>
<tr>
<td>UDP-GlcUA (M3G)</td>
<td>0.64 ± 0.05</td>
<td>2696 ± 104</td>
</tr>
<tr>
<td>UDP-GlcUA (M6G)</td>
<td>0.56 ± 0.06</td>
<td>378 ± 15</td>
</tr>
</tbody>
</table>

$^aP < 0.05$; comparisons for each cofactor with and without BSA (i.e., across rows).

$^bP < 0.01$; comparisons for each cofactor with and without BSA (i.e., across rows).

$^cP < 0.01$; UDP-GlcUA compared with UDP-Glc (i.e., down columns).

The derived kinetic constants for morphine glycosidation generated in the absence and presence of BSA (2% w/v) with cofactor (UDP-Glc or UDP-GlcUA) as the variable substrate are shown as the mean ± S.D. of four replicates with pooled HLM.
Cofactor Docking Experiments and Activities of Mutant UGT2B7 Proteins. Docking experiments with the UGT2B7 homology model demonstrated that both UDP-Glc and UDP-GlcUA bind within the same domain of the C terminus of UGT2B7, and binding interactions of the UDP moiety are cofactor independent. However, residues involved in the binding of the sugar differed; Asp398 and Glu399 interact with glucose, whereas Asn402 and Tyr33 hydrogen bond to the glucuronic acid moiety of UDP-GlcUA (Fig. 6). The anomeric carbons of both cofactors are within 5 Å of His35 (Fig. 6), the putative catalytic base involved in the glucuronidation reaction.

The Y33F, Y33L, D398A, D398L, Q399A, Q399L, N402A, N402L, N402H, and N402Q mutants were generated by site-directed mutagenesis on the basis of the docking experiments referred to above. The Tyr residue present at position 33 of wild-type UGT2B7 was substituted with the smaller, neutral Leu and with Phe, which is of similar size to Tyr but is nonpolar. The polar Asp and Asn residues at positions 398 and 402, respectively, of wild-type UGT2B7 were substituted with Ala (small and nonpolar) and Leu, which is of similar size to Asp and Asn but is nonpolar. Because the docking studies implicated Asn402 in the binding of the carboxylate group of UDP-GlcUA, this residue was additionally substituted with the chemically related amino acid Gln in a further attempt to differentiate residues involved in the binding of UDP-GlcUA and UDP-Glc. In addition, Miley et al. (2007) predicted a role for Asn378 in the binding of the carboxylate group of UDP-GlcUA based on docking studies with the X-ray crystal structure of the C-terminal domain of UGT2B7. Furthermore, His occurs at the equivalent position to Asn378 in all UGT1A subfamily proteins. Thus, the N378A, N378L, N378Q, and N378H mutants were also generated.

Mutant UGT2B7 enzymes were expressed in HEK293T cells. All mutants expressed at a similar level to that of wild-type UGT2B7, as demonstrated by Western blotting with an anti-UGT2B7 antibody (Fig. 7). However, all of the 378-, 398-, 399-, and 402- mutants and Y33L lacked activity toward morphine, with either UDP-Glc or UDP-GlcUA as cofactor. Activity was observed only with wild-type UGT2B7 and the Y33F mutant. In contrast to the loss of activity arising from substitution of Tyr with the dissimilar Leu at position 33, the conserved Tyr → Phe substitution resulted in only minor changes in glucosidation activities. At morphine concentrations of 1, 5, and 10 mM, rates of M3G formation ranged from 53 to 144 and 62 to 185 pmol/min/mg for wild-type UGT2B7 and the Y33F mutant, respectively, whereas rates of M6G formation ranged from 9 to 27 and 12 to 35 pmol/min/mg for wild-type UGT2B7 and the Y33F mutant, respectively. Rates of M-3-glucoside formation by the wild-type UGT2B7 and the Y33F mutant ranged from 11 to 39 and 7 to 29 pmol/min/mg, respectively.

**Discussion**

HLM supplemented with UDP-GlcUA alone formed both M3G and M6G. The ratios of the mean CL_int values for M3G to M6G (i.e. ~5.5), in both the presence and absence of BSA (2%
a potentially important consideration for in vitro metabolism of any given compound should be performed in contributions of glucuronidation and glucosidation to the more consistent with the in vivo data of Chen et al. (2003).

Glucoside formation were lower than those of M6G, indicating a lesser role for glucosidation in morphine metabolism. This is possibly because of enzyme conformational changes that occur upon aglycone binding, possibly because of enzyme conformational changes that occur upon aglycone binding.

The observation of negative cooperative kinetics with UDP-GlcUA and UDP-Glc warrants comment. Although the mechanism for the negative cooperative cofactor kinetics has not been investigated directly, “atypical” aglycone glucuronidation kinetics, including negative cooperativity, occurs not uncommonly (Miners et al., 2010). Indeed, negative cooperative-like kinetics have been reported for morphine glucuronidation by HLM (Miners et al., 1988) and by recombinant UGT2B7 (Stone et al., 2003). Atypical glucuronidation data with UGT2B7 as the enzyme source have been modeled previously by a mechanistic model that assumes two interacting aglycone binding sites (Stone et al., 2003; Uchaipichat et al., 2004, 2008). It is possible that the two substrate binding sites arise from dimerization. This model also provides the basis for two cofactor binding domains.

Tang et al. (2003) previously observed mutual competitive inhibition by UDP-Glc and UDP-GlcUA in the acyl glucosidation and glucuronidation of an endothelin ET_2 antagonist by HLM and recombinant UGT2B7. K_m and K_i values for each cofactor-enzyme source combination were both ~0.6 mM, which is similar to the S_{50} found here for UDP-GlcUA but lower than our value for UDP-Glc (i.e. 2 mM). Subsequent experiments by the same group with diclofenac glucuronidation and glucosidation by UGT2B7 have also been reported (Mackenzie et al., 2003; Toide et al., 2004; Tang and Ma 2005), suggesting that the selectivity and binding affinity of UDP-sugars may be aglycone dependent, possibly because of enzyme conformational changes that occur upon aglycone binding. Although this may provide an explanation for the differing results between studies, most, but not all, kinetic investigations of the glucuronidation reaction suggest that UDP-GlcUA binding to the UGT enzyme occurs first (Luukkanen et al., 2005). Furthermore, inhibition of morphine 3- and 6- glucuronidation in the absence and presence of BSA were an order of magnitude higher, ranging from 1.63 to 2.49 mM. Last, the CL_{int} values for M-3-glucoside formation in the presence of mixed cofactor (± BSA) were lower than those measured in the presence of UDP-Glc alone primarily due to an ~85% reduction in V_{max}, whereas V_{max} values for the morphine glucuronidation pathways did not differ significantly between the single (i.e., UDP-GlcUA) and mixed cofactor experiments. This presumably arises from the preferential binding of UDP-GlcUA to UGT2B7.

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Several lines of evidence suggest that UDP-GlcUA binds with higher affinity to UGT2B7, at least with morphine as the aglycone, than does UDP-Glc. First, S_{50} values obtained with UDP-GlcUA as the variable substrate were close in value for both the M3G and M6G pathways and 70 to 83% lower (depending on the presence or absence of BSA; discussed later) than the S_{50} for UDP-Glc, which was measured with respect to M-3-glucoside formation. Second, K_i values for UDP-GlcUA inhibition of M-3-glucosidation (± BSA) were 0.15–0.18 mM, respectively, whereas K_i values for UDP-Glc

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**Fig. 6.** Docking of UDP-glucose and UDP-glucuronic acid (as the carboxylate anion) in the UGT2B7 homology model. The catalytic base (His35; cyan) and Asn378 (green) are shown as reference points. (A) Tyr33 and Asn402 (green) hydrogen bond with the glucuronic acid moiety of UDP-GlcUA. (B) Asp398 and Gln (green) hydrogen bond to the glucose moiety of UDP-Glc.

**Fig. 7.** Immunoblots of lysates of HEK293 cells expressing wild-type and mutant UGT2B7 proteins (50 μg). No band was detected with untransfected HEK293 cells.
glucosidation studies with recombinant UGT enzymes expressed in insect cells, as used by Tang and colleagues (Tang et al., 2003; Tang and Ma, 2005), may be complicated by the expression of an endogenous glucosyltransferase (see Discussion).

Given the known major role of UGT2B7 in the 3- and 6-glucuronidation of morphine, kinetic studies were performed in the presence of BSA. As noted in the Introduction, BSA enhances the glucuronidation of UGT2B7 substrates via a reduction in $K_m$ (Rowland et al., 2007; Manevski et al., 2011; Walsky et al., 2012). In both the single and mixed (1:1) cofactor experiments, BSA (2% w/v) decreased the $K_m/S_{50}$ values for morphine 3- and 6-glucuronidation and morphine 3-glucosylation by HLM to a similar extent (77–90% reduction) with little or no effect on $V_{max}$. Similarly, BSA had only a minor effect (<30% change) on the $V_{max}$ values for UDP-GlcUA and UDP-Glc. Although there was no effect of BSA on the $S_{50}$ values for UDP-Glc, there was a modest reduction (by 40%) in the $S_{50}$ value for UDP-GlcUA. Available evidence suggests that BSA sequesters inhibitory long-chain unsaturated fatty acids released by membranes during the course of an interaction, thereby reducing the $K_m$ values of the aglycone substrates of UGT2B7 and certain other UGT enzymes (Rowland et al., 2007, 2008). The effect of BSA on the $S_{50}$ for UDP-GlcUA suggests that fatty acids may also influence the binding of this cofactor but to a lesser extent than the binding of the aglycone.

Consistent with the observation that M-3-glucoside formation was enhanced by BSA, experiments with recombinant UGT enzymes of the IA and IB subfamilies demonstrated that only UGT2B7 glucosidates morphine. The UGT enzyme activity studies further confirmed the major contribution of UGT2B7 to morphine 3- and 6-glucuronidation. Moreover, fluconazole (a selective UGT2B7 inhibitor) and AZT (a selective UGT2B7 substrate) inhibited M-3-glucoside formation by HLM, whereas hecogenin and niflumic acid, which selectively inhibit UGT1A4 and UGT1A9/1A1/2B15, respectively, did not. Interestingly, of the UGT1A and UGT2B enzymes expressed in HEK293 cells, only UGT2B7 glucosidated morphine, whereas Supersomes expressing UGTs 2B4, 2B7, 2B15, and 2B17 all formed M-3-glucoside. By contrast, M3G and M6G formation was observed only with Supersomes expressing UGT2B7 (major) and UGT2B4 (minor). Importantly, untransfected Supersomes exhibited high M-3-glucosidation activity, but when corrected for endogenous glucosidation activity, significant M-3-glucoside formation occurred with just UGT2B7. Apart from morphine, the enzyme present in Supersomes has the capacity to glucosidate a structurally diverse range of xenobiotics that are metabolized by glucuronidation (manuscript in preparation).

Molecular modeling and site-directed mutagenesis studies were used to identify the amino acids involved in the binding of the sugar moieties of UDP-Glc and UDP-GlcUA to the cofactor binding domain of UGT2B7. Docking experiments with a UGT2B7 homology model identified potential hydrogen bonding interactions between Asp398 and Glu399 with the hydroxyl groups of glucose and Asn402 and Tyr33 with the carboxylate group of glucuronic acid. Based on the X-ray crystal structure of the C-terminal half of UGT2B7, Miley et al. (2007) further predicted a role for Asn378 in the binding of the carboxylate group of UDP-GlcUA. In the UGT1A subfamily, His occurs at the position equivalent to Asn378 in UGT2B7. Thus, the Y33F, Y33L, N378A, N378L, N378Q, N378H, D398A, D398L, Q399A, Q399L, N402A, N402L, N402H, and N402Q mutants were generated by site-directed mutagenesis (see Results for the rationale underlying the substitutions). Although all mutants were expressed to a similar level in HEK293 cells, morphine glucuronidation and glucosidation activity was observed only with wild-type UGT2B7 and the Y33F mutant. Likewise, 4-MU glucuronidation activity was observed only with wild-type UGT2B7 and the Y33F mutant (data not shown), whereas rates of 4-MU glucuronidation were very low (<1 pmol/min/mg) with all mutants and wild-type UGT2B7. Collectively, these data indicate that Asn378, Asp398, Glu399, and Asn402 are essential for the binding of both UDP-GlcUA and UDP-Glc. An important role for Tyr33, located close to the putative active site of UGT2B7 (Lewis et al., 2011), also seems likely because the dissimilar Tyr→Leu substitution resulted in loss of activity, whereas the conserved Y33F mutation had little or no effect on glycosidation activity. Mutations in this region of UGT1A9 have similarly been demonstrated to have a profound effect on xenobiotic glucuronidation (Korpraserthaworn et al., 2012).

As noted in the Introduction, a number of structurally diverse compounds are known to undergo both glucuronidation and glucosidation. In addition to morphine, UGT2B7 has also been implicated in the glucuronidation and glucosidation of an experimental ETA antagonist, AS-3201, hydoexoxycholic acid, and ibuprofen, variably forming phenolic, acyl, and N-glycosides (Mackenzie et al., 2003; Tang et al., 2003; Toide et al., 2004; Buchheit et al., 2011). However, other enzymes of the UGT2B and UGT1A subfamilies also catalyze AS-3201 glucosidation while UGT1A1 catalyzes the glucuronidation and glucosidation of bilirubin (Senafi et al., 1994; Toide et al., 2004). These data suggest that glucuronidation and glucosidation, catalyzed by common UGT enzymes, may occur as parallel metabolic pathways. However, some drugs that are not glucuronidated in humans undergo glucosidation, including amobarbital, phenobarbital, some sulfonamides, and 5-aminosalicylic acid (Tjornelund et al., 1989; Tang 1990; Meech et al., 2012). The enzyme(s) responsible for the formation of these glucosides appear not to have been elucidated.

In summary, the present study has demonstrated that morphine 3- and 6-glucuronidation and morphine 3-glucosidation occur as complementary metabolic pathways catalyzed by a common enzyme (UGT2B7). In vitro kinetic data from experiments with the individual cofactors appear to overestimate the contribution of glucosidation to morphine elimination, whereas data generated in the presence of both UDP-GlcUA and UDP-Glc are consistent with in vivo observations, presumably because of the higher binding affinity UDP-GlcUA to UGT2B7. Molecular modeling and site-directed mutagenesis confirm the importance of Tyr33, Asn378, Asp398, Glu399, and Asn402 in the binding of UDP-GlcUA and UDP-Glc to UGT2B7, consistent with a common cofactor binding domain.

**Authorship Contributions**
- ** Participated in research design:** Chau, Mackenzie, Miners.
- **Conducted experiments:** Chau, Elliot, Lewis, Burns.
- **Performed data analysis:** Chau, Lewis, Johnston, Miners.
- **Wrote or contributed to the writing of the paper:** Chau, Miners.
References


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