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The glucuronidation of Δ^4-3-keto C19- and C21- hydroxy-steroids by human liver microsomal and recombinant UDP-glucuronosyltransferases (UGT): 6α- and 21-hydroxyprogesterone are selective substrates for UGT2B7.

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Running title: UGT enzyme selectivity of Δ^4-3-keto hydroxy-steroid glucuronidation

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Non-standard abbreviations:

HEK293, human embryo kidney 293 cells; HLM, human liver microsomes; HPLC, high performance liquid chromatography; 6β-OHAD, 4-androsten-6β-ol-3, 17-dione; 7α-OHAD, 4-androsten-7α-ol-3, 17-dione; TST, testosterone; 6α-OHP, 6α-hydroxyprogesterone; 6β-OHP, 6β-hydroxyprogesterone; 11α-OHP, 11α-

hydroxyprogesterone; 11β-OHP, 11β-hydroxyprogesterone; 16α-OHP, 16α-hydroxyprogesterone; 17α-OHP, 17α-hydroxyprogesterone; 21-OHP, 21-hydroxyprogesterone; 4-MU, 4-methylumbelliferone; 4-MUG, 4-methylumbelliferone-β-D-glucuronide;UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid.

ABSTRACT

The stereo- and regio- selective glucuronidation of ten Δ^4-3-keto mono hydroxylated androgens and pregnanes was investigated in order to identify UGT enzyme selective substrates. Kinetic studies were performed using human liver microsomes (HLM) and a panel of 12 recombinant human UGTs as the enzyme sources. Five of the steroids, which were hydroxylated in the 6β-, 7α-, 11β- or 17α- positions, were not glucuronidated by HLM. Of the remaining compounds, comparative kinetic and inhibition studies indicated that 6α- and 21- hydroxyprogesterone (6α- and 21- OHP) were glucuronidated selectively by human liver microsomal UGT2B7. 6α-OHP glucuronidation by HLM and UGT2B7 followed Michaelis-Menten kinetics, whereas 21-OHP glucuronidation by these enzyme sources exhibited positive cooperativity. UGT2B7 was also identified as the enzyme responsible for the high affinity component of human liver microsomal 11α- hydroxyprogesterone (11α-OHP) glucuronidation. In contrast, UGT2B15 and UGT2B17 were the major forms involved in human liver microsomal testosterone 17β-glucuronidation and the high affinity component of 16α- hydroxyprogesterone (16α-OHP) glucuronidation. Activity of UGT1A subfamily enzymes towards the hepatically glucuronidated substrates was generally low, although UGT1A4 and UGT1A9 contribute to the low affinity components of microsomal 16α-OHP and 11α-OHP glucuronidation, respectively. Interestingly, UGT1A10, which is expressed only in the gastrointestinal tract, exhibited activity towards most of the glucuronidated substrates. The results indicate that 6α- and 21- OHP may be used as selective 'probes' for human liver microsomal UGT2B7 activity and, taken together, provide insights into the regio- and stereo-selectivity of hydroxy-steroid glucuronidation by human UGTs.

UDP-Glucuronosyltransferase (UGT) enzymes catalyze the covalent linkage of glucuronic acid, derived from the co-factor UDP-glucuronic acid (UDPGA), to typically lipophilic substrates bearing a suitable acceptor group, most commonly hydroxyl, carboxylic acid, or amine. Given the ability of UGT to metabolize such commonly occurring chemical features, conjugation with glucuronic acid ('glucuronidation') assumes importance for the elimination and detoxification of drugs, environmental chemicals, and endogenous compounds (Miners and Mackenzie, 1991). UGTs have been classified in two families, UGT1 and UGT2, based on the sequence identity of the encoded proteins (Mackenzie et al., 2005). Of the nineteen human UGT proteins identified to date, thirteen appear to exhibit significant activity towards drugs, environmental chemicals and/or endogenous compounds; UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17 and 2B28 (Miners et al., 2004). The individual UGTs possess distinct, albeit overlapping, substrate selectivities and differ in terms of regulation of expression. For example, age, diet, disease states, induction and inhibition by coadministered chemicals, ethnicity, genetic polymorphism, and hormonal factors are all known to influence UGT activity (Miners and Mackenzie, 1991; Miners et al., 2001). Furthermore, differences occur in regulation of expression; while most UGTs are expressed in liver, UGT 1A7, 1A8 and 1A10 are localized in the gastrointestinal tract (Mojarrabi and Mackenzie, 1998; Tukey and Strassburg, 2000).

Given these features of UGTs, recent attention has focused on identifying enzyme-selective probes, that is compounds selectively glucuronidated by a single UGT. The availability of selective substrates is important for the reaction phenotyping of

glucuronidation pathways, and for evaluating the selectivity of drug-drug interactions and the functional significance of *UGT* genetic polymorphism (Court 2005; Miners et al., 2006). The majority of studies conducted to date have focused on the characterization of xenobiotic probe substrates for UGT enzymes. However, there is evidence with both cytochromes P450 (CYP) and UGT demonstrating that the stereo- and regio- selective metabolism of steroids provides an alternative approach to the identification of enzyme–selective pathways.

The hydroxylation of C18-, C19- and C21- steroids is known to occur at almost all non-bridgehead carbon atoms in humans, although certain positions may be favored depending on the structure of the substrate (Setchell et al., 1976; Hobkirk, 1979). In addition, hydroxylation may be stereoselective, leading to the formation of α-and β-isomers. Many of these hydroxy-steroids are excreted in urine as the glucuronide conjugates (Fotherby and James, 1972; Setchell et al., 1976; Musey et al., 1979). As noted above, regio- and stereo- selective steroid hydroxylation may be used for identifying CYP enzyme selective pathways. For example, it is well established that testosterone 6β- hydroxylation is catalyzed selectively by CYP3A (Mei et al., 1999). Similarly, UGT1A and UGT2B enzymes appear to differentially contribute to the glucuronidation of hydroxylated derivatives of C18- (estrogens), C19- and C21-steroids (Jin et al., 1997; Turgeon et al., 2001; Kuuranne et al., 2003; Lepine et al., 2004).

Δ^4-3-Keto- hydroxylated C19- and C21- steroids provide a convenient series of compounds for investigating the regio- and stereo- selectivity of hydroxy-steroid glucuronidation. Numerous compounds are available commercially and the Δ^{4-}3-keto-

moiety provides a chromophore suitable for the uv detection of products by straightforward HPLC methods. This study investigated the UGT enzyme selectivity of a series of 6α-, 6β-, 7α-, 11α-, 11β-, 16α-, 17α-, 17β- and 21- mono hydroxylated derivatives of C19- and C21- steroids bearing the Δ^4- 3-keto- function (Fig 1). While the stereo- and regio- selectivity of hydroxy-steroid glucuronidation by recombinant UGTs and human liver microsomes was undertaken primarily to identify pathways that may be selective for hepatic UGT enzymes, the work provides further insights into the structural determinants of substrates that confer UGT enzyme selectivity.

MATERIALS AND METHOD

Materials

6α-Hydroxyprogesterone (6α-OHP), 6β-hydroxyprogesterone (6β-OHP) and 4-androsten-7α-ol-3, 17-dione (7α-OHAD) were purchased from Steraloids (Wilton, NH). 4-Androsten-6β-ol-3, 17-dione (6β-OHAD), testosterone (TST), 11α-hydroxyprogesterone (11α-OHP), 11β-hydroxyprogesterone (11β-OHP), 16α-hydroxyprogesterone (16α-OHP), 17α-hydroxyprogesterone (17α-OHP), 21-hydroxyprogesterone (21-OHP), 4-methylumbelliferone (4-MU), 4-methylumbelliferone-β-D-glucuronide (4-MUG), alamethicin (from *Trichoderma viride*), β-glucuronidase (from *E. coli*) and UDP-glucuronic acid (UDPGA; sodium salt) were purchased from Sigma-Aldrich (Sydney, Australia). Solvents and other reagents used were of analytical reagent grade.

Methods

Human liver microsomes and recombinant UGTs

Microsomes were prepared from human liver tissue by differential centrifugation, as described by Bowalgaha *et al.* (2005).The five human livers used in this study (H7, H12, H13, H29 and H40) were obtained from the human liver 'bank' of the Department of Clinical Pharmacology, Flinders Medical Centre. Approval for the use of human liver tissue for *in vitro* drug metabolism studies was granted by the Clinical Investigation Committee of Flinders Medical Centre. Human liver microsomes (HLM) used to investigate hydroxy-steroid glucuronidation were activated with alamethicin (50 μg/mg microsomal protein), as described by Boase and Miners (2002).

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Human UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17 and 2B28 cDNAs were stably expressed in a human embryonic kidney cell line (HEK293) according to Stone *et al.* (2003) and Uchaipichat *et al.* (2004). After growth to at least 80% confluency, cells were harvested and washed in phosphate-buffered saline. Cells were subsequently lysed by sonication using a Vibra Cell VCX 130 Ultrasonics Processor (Sonics and Materials, Newtown, CT). Cells expressing UGT1A enzymes were sonicated with four 'bursts' each lasting 2 sec, separated by 1min with cooling on ice. Cells expressing UGT2B enzymes were treated using the same method, except sonication was limited to 1sec bursts. Lysates were centrifuged at 12,000 g for 1 min at 4°C, and the supernatant fraction was separated and stored in phosphate buffer (0.1 M, pH 7.4) at –80°C until use. Expression of each UGT was demonstrated by immunoblotting with a commercial UGT1A antibody (BD Gentest, Woburn, MA, USA) or a non-selective UGT antibody (raised against purified mouse Ugt) (Uchaipichat *et al.* 2004) and activity measurements (see below).

Prior to incubations, activities of recombinant UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17 and 2B28 were confirmed using the non-selective substrate 4-methylumbelliferone (4-MU). For all enzymes, except UGT2B4 and UGT2B28, the conversion of 4-MU to 4-MUG was measured according to the fluorometric procedure of Miners *et al.* (1988), as modified by Uchaipichat *et al.* (2004). The concentration of 4-MU present in incubations corresponded to the known K_m (or S_{50}) for each enzyme (Sorich *et al.* 2002; Uchaipichat *et al.* 2004). Given the lower specific activities of UGT 2B4 and 2B28, 4-MU glucuronidation activity (at 1000 μM) was confirmed using a radiometric thin layer chromatographic procedure

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(Jin *et al.*, 1997). The activity of UGT1A4 was demonstrated using lamotrigine as substrate, at the approximate K_m for this substrate (1500 μM), according to the method of Rowland *et al.* (2006).

Glucuronidation of hydroxy-steroids by human liver microsomes and recombinant UGTs

Hydroxy-steroid glucuronidation assays

Screening for hydroxy-steroid glucuronidation by pooled HLM (equal amounts of microsomal protein from the five livers used in kinetic studies) was performed at 37˚C in a total incubation volume of 200 μl. Incubations contained microsomal protein (1 mg/ml), substrate (3 concentrations in the range 5- 250 μM), phosphate buffer (0.1 M, pH 7.4), $MgCl_2$ (4 mM) and UDPGA (5 mM). Reaction mixtures were pre-incubated at 37°C for 5 min prior to initiation of the reaction by addition of co-factor (UDPGA). Reactions were carried out in air at 37°C (shaking water bath) for 120 min, and then terminated by the addition 2 μl of perchloric acid (11.6 M) and cooling on ice. Mixtures were vortex mixed and subsequently centrifuged (4000 g for 10 min). A 30 μl aliquot of the supernatant fraction was analysed by HPLC. Like experiments with HLM, the ability of recombinant UGTs to glucuronidate hydroxy-steroids was determined for three substrate concentrations in the range 5 - 250 μM. Stock solutions of all hydroxy-steroids were prepared in methanol; the final concentration of solvent in incubations was 1% v/v, which has a negligible effect on UGT enzyme activities (Uchaipichat *et al.* 2004). Activity screening experiments with recombinant UGTs were performed with 1 mg/ml HEK cell lysate protein expressing the UGT of interest for 120 min.

Where activity was observed, kinetic studies were performed with microsomes from five separate human livers and the individual recombinant UGTs shown to glucuronidate each hydroxy-steroid in activity screening experiments. Kinetic experiments were performed in duplicate for 10-14 substrate concentrations spanning the K_m (or S_{50}). Linearity of product formation with respect to incubation time and protein concentration for HLM and expressed UGT enzymes was established for all substrates investigated prior to kinetic analysis. Optimized incubation conditions for each hydroxy-steroid investigated are given in Table 1.

Inhibition of hydroxy-steroid glucuronide formation by zidovudine (AZT)

Where hydroxy-steroid glucuronidation appeared to involve UGT2B7, inhibition studies were performed with pooled HLM (equal amounts of microsomal protein from 5 livers; see above) and UGT2B7 in the presence and absence of AZT, a known selective substrate for UGT2B7. Inhibition experiments were performed at an AZT concentration of 5mM (which is approximately 5-fold the K_m for AZT glucuronidation by HLM; Boase and Miners 2002), and with the substrate concentration corresponding to the known K_m (with HLM as the enzyme source). When kinetics described by the 2 enzyme Michaelis-Menten or Hill equations were observed, AZT inhibition experiments were carried out at a high and a low substrate concentration (see subsequent results) using the incubation conditions shown in Table 1.

Measurement of hydroxy-steroid glucuronide formation

Hydroxy-steroid glucuronidation was quantified by reversed phase HPLC. The HPLC system employed (Agilent Technologies, Sydney, Australia) consisted of a gradient

solvent delivery system, UV detector and auto sampler. The instrument was fitted with a Nova-Pak C18 (3.9 mm (id) x 150 mm, 4 μm particle size) analytical column (Waters Corporation, Milford, MA, USA). HPLC conditions for each hydroxy-steroid are shown in Table 2. Analytes were eluted at a flow rate of 1 ml/min and detected by UV absorption at 241 nm. The Δ^4-3-keto ring-A structure of hydroxy-steroids provides a convenient chromophore for analysis of product formation by UV absorption at 241nm. Chromatography was performed at 25°C.

The identity of hydroxy-steroid glucuronide formed in incubations with HLM and expressed UGT enzymes was confirmed by hydrolysis with β-glucuronidase (from *E. coli)*, acid hydrolysis and comparison with blank incubations (which excluded UDPGA). Concentrations of TST glucuronide in incubations were determined by comparison of the peak areas with a standard curve constructed from an authentic TST glucuronide standard. For the remaining substrates, concentrations of hydroxy-steroid glucuronides in incubations were determined by comparison of the peak areas with a standard curve constructed from known concentrations of each hydroxy-steroid. Response factors of testosterone glucuronide and testosterone were shown to be equivalent. Nevertheless, V_{max} values for other hydroxy-steroid glucuronides should be considered 'apparent'. Calibration standards were treated in the same manner as incubation samples. The lower limit of quantification for each hydroxy-steroid assay, defined as five times background absorbance, was 0.04 μM hydroxy-steroid glucuronide.

Within-day overall assay reproducibility for each hydroxy-steroid assay was assessed by measuring the rate of glucuronide formation in eight separate incubations of

pooled human liver microsomes. Assay reproducibility was assessed at two (low and high) substrate concentrations for each assay. For all hydroxy-steroids tested, within day coefficients of variation were < 3.6% and < 5.2 % at the low and high concentrations, respectively.

Data Analysis

Kinetic constants for steroid glucuronidation were determined by fitting untransformed experimental data to equations for the single and 2 enzyme Michaelis-Menten equations and the Hill equation (where indicated) using the non–linear least squares fitting program EnzFitter version 2.0 (Biosoft, Cambridge, UK). Goodness of fit was assessed from comparison of the parameter SE of fit, coefficient of determination (r^2), and F-statistic.

RESULTS

Hydroxy-steroid glucuronidation by human liver microsomes and recombinant UGTs

The hydroxy-steroids shown in Fig 1 were screened for glucuronide formation by pooled HLM. Only TST, 6α-OHP, 11α-OHP, 16α-OHP and 21-OHP were glucuronidated at a rate > 0.5 pmol/min.mg by HLM (data not shown). For example, glucuronidation activities ranged from 66 pmol/min.mg for 16α -OHP to 1290 pmol/min.mg for 11α-OHP at a substrate concentration of 50 μM. Thus, the glucuronidation of TST, 6α-OHP, 11α-OHP, 16α-OHP and 21-OHP by both HLM and recombinant UGTs was investigated further. Kinetic parameters for hydroxy-steroid glucuronidation by HLM and UGTs are summarized in Table 3. Eadie-Hofstee plots for hydroxy-steroid glucuronidation by microsomes from a representative liver (H13) are shown in Fig 2.

As indicated in Methods, each hydroxy-steroid was screened for glucuronidation activity by twelve recombinant human UGTs. It was found that UGT 1A3, 1A4, 1A7, 1A8, 1A9, 1A10, 2B7, 2B15 and 2B17 variably contributed to the glucuronidation of the hydroxy-steroids investigated (see subsequent results). Representative Eadie-Hofstee plots for hydroxy-steroid glucuronidation by recombinant human UGT 2B7 and 2B17 are shown in Fig 3. UGT 1A1, 1A6, 2B4, and 2B28 did not glucuronidate any of the substrates investigated here, at least to the limit of detection of the assays employed. It should be noted that where activity was observed with UGT 1A7, 1A8 and 1A10, kinetic studies were not performed since these enzymes are not expressed in liver.

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TST glucuronidation

TST glucuronidation exhibited Michaelis-Menten kinetics in all five livers investigated (Fig 2A and Table 3), with respective mean (±SD) derived K_m and V_{max} values of 5.6 ± 0.96 μM and 196 ± 113 pmol/min.mg. Seven recombinant enzymes (UGT 1A3, 1A4, 1A8, 1A10, 2B7, 2B15 and 2B17) glucuronidated TST. Rates of TST glucuronidation (at 100 μM) by recombinant UGT 1A10, 2B15, and 2B17 were in the range 5.0 – 475 pmol/min.mg, while those of UGT 1A3, 1A4, 1A8 and 2B7 were <2.5 pmol/min.mg. TST glucuronidation by UGT 2B15 and 2B17 (Fig 3A) followed Michaelis-Menten kinetics, with respective K_m values of 5.7 and 3.8 μM (Table 3).

6α-OHP glucuronidation

6α-OHP glucuronidation similarly exhibited Michaelis-Menten kinetics in all five livers investigated (Fig 2B, Table 3), with derived mean K_m and V_{max} values of 94 ± 22 μM and 1828 ± 743 pmol/min.mg, respectively. Recombinant UGT 1A3, 1A10 and 2B7 glucuronidated 6α-OHP. The rate of 6α-OHP glucuronidation by UGT2B7 at a substrate concentration of 250 μM was 1210 pmol/min.mg, whereas UGT 1A3 and 1A10 exhibited rates <2.5 pmol/min.mg. The glucuronidation of 6α-OHP by UGT2B7 followed Michaelis-Menten kinetics (Fig 3B), with respective K_m and V_{max} values of 55 μM and 269 pmol/min.mg. AZT inhibition experiments were conducted at a 6α-OHP concentration of 100 μM (the mean approximate K_m for the HLM catalyzed reaction). AZT (5 mM) inhibited human liver microsomal 6α-OHP glucuronidation by 74%, and UGT2B7 catalyzed 6α-OHP glucuronidation by 83%.

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11α-OHP glucuronidation

Non-Michaelis-Menten kinetics were observed for 11α-OHP glucuronidation by microsomes from four of the five livers investigated (Table 3). Data for microsomes from H12, H13, H29 and H40 were well described by the 2-enzyme Michaelis-Menten equation (Fig 2C), with apparent K_m values of 3.8 ± 1.8 μM and 125 ± 172 μM for the high and low affinity components, respectively. In contrast, data for microsomes from liver H7 were consistent single enzyme Michaelis-Menten kinetics (K_m 11 μM; Table 3). Recombinant UGT 1A3, 1A4, 1A7, 1A9, 1A10 and 2B7 glucuronidated 11α-OHP. The kinetics of 11α-OHP glucuronidation by recombinant UGT 1A9 and 2B7 were characterized on the basis of activities >2.5 pmol/min.mg at a substrate concentration of 250 μM. Conversion of 11α-OHP to its glucuronide by UGT1A9 (K_m 93 μM) and UGT2B7 (K_m 6.9 μM; Fig 3C) followed single enzyme Michaelis-Menten kinetics. AZT inhibition experiments were performed at 11α-OHP concentrations of 4 μM and 100 μM. (Substitution of the mean K_m and V_{max} values for the high and low affinity components of 11α-OHP glucuronidation by HLM (Table 3) in the 2 enzyme Michaelis-Menten equation indicates that the *high* affinity enzyme is responsible for 89% of activity at substrate concentration of 4 μM and 52% of activity at a substrate concentration of 100 μM.) AZT (5mM) inhibited human liver microsomal 11α-OHP glucuronidation by 75% at the lower substrate concentration, and by 29% at the higher substrate concentration.

16α-OHP glucuronidation

16α-OHP glucuronidation by HLM exhibited biphasic kinetics for all five livers investigated (Fig 2D, Table 3). Fitting of experimental data to the 2-enzyme Michaelis-Menten equation gave mean (±SD) apparent K_m values for the high and

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low affinity components of 4.0 ± 1.5 μM and 130 ± 103 μM, respectively, and V_{max} values of 49 ± 29 pmol/min.mg and 113 ± 46 pmol/min.mg, respectively. UGT 1A4, 1A10, 2B7, 2B15 and 2B17 all glucuronidated 16α-OHP. Kinetic data for 16α-OHP glucuronidation by UGT1A4 (K_m 73 μM), UGT2B7 (K_m 211 μM), 2B15 (K_m 3.0 μM) and 2B17 (K_m 3.0 μM; Fig 3D) all followed hyperbolic kinetics. AZT inhibition experiments were conducted at 16α-OHP concentrations of 20 μM and 100 μM. Substitution of the mean K_m and V_{max} values for the high and low affinity components of 16α-OHP glucuronidation by HLM (Table 3) in the 2 enzyme Michaelis-Menten equation indicates that *low* affinity enzyme is responsible for 29% and 51% of activity at substrate concentrations of 20 μM and 100 μM, respectively. AZT (5mM) inhibited human liver microsomal 16α-OHP glucuronidation by 30% and 40% at the low and high substrate concentrations, respectively.

21-OHP glucuronidation

21-OHP glucuronidation by HLM was best described by the Hill equation with positive cooperativity (autoactivation) in four of the five livers studied, with a mean S_{50} value of 34 ± 12 μM, Hill coefficient (n) of 1.22 ± 0.14, and V_{max} of 217 ± 54 pmol/min.mg (Table 3, Fig 2E). In contrast, data for microsomes from liver H29 were best described by the single enzyme Michaelis-Menten equation (Table 3). Of the recombinant UGTs screened, UGT 1A10 and 2B7 glucuronidated 21-OHP. Glucuronidation of this compound by UGT2B7 exhibited sigmoidal kinetics which were modeled using the Hill equation (Fig 3E). Derived S_{50} and n and values were 19 μM and 1.16, respectively. AZT inhibition experiments were conducted at 21-OHP concentration of 20 μM (the approximate S_{50}). AZT inhibited human liver microsomal

and UGT2B7 catalyzed 21-OHP glucuronide formation by 86% and 100%, respectively.

DISCUSSION

The liver is generally considered to be the main organ responsible for the glucuronidation of circulating hydroxy-steroids, although extrahepatic UGTs are presumably of importance as a 'local' detoxification mechanism (Hum et al., 1999). This work sought primarily to identify substrate 'probes' for human hepatic UGT enzymes using a series of Δ^4-3-keto hydroxy-steroids. Of the compounds screened, TST and 6α-, 11α-, 16α-, and 21- OHP were glucuronidated by HLM, whereas 6β- and 7α- OHAD and 6β-, 11β- and 17α- OHP lacked measurable glucuronidation activity. Comparative kinetic studies with a panel of recombinant enzymes indicated that, of the hepatically expressed enzymes, UGT 2B7, 2B15 and 2B17 were the major contributors to the glucuronidation of the compounds metabolized by HLM. UGT 1A3, 1A4, 1A7, 1A8, 1A9 and 1A10 variably glucuronidated the substrates metabolized by HLM. However, with some exceptions, activity of the UGT1A subfamily enzymes was generally low (<2.5 pmol/min.mg). UGT1A10, which is expressed solely in the gastrointestinal tract, significantly glucuronidated TST and 16α- and 21- OHP. UGT1A9 appeared to be the enzyme responsible for the low affinity component of human liver microsomal 11α-OHP glucuronidation, and a contribution of UGT1A4 to the low affinity component of 16α-OHP glucuronidation cannot be discounted.

Importantly, comparison of kinetic and AZT inhibition data using HLM and recombinant UGTs as the enzyme sources indicated that 6α- and 21- OHP were glucuronidated selectively by UGT2B7. In addition, UGT2B7 appeared to be the high- and low- affinity enzymes responsible for human liver microsomal 11α-OHP and 16α-OHP glucuronidation, respectively. Thus, 6α- and 21- OHP may serve as

substrate probes for human hepatic UGT2B7 activity in vitro. Of these, 6α-OHP has two advantages; higher activity (approximately 10-fold higher V_{max} with HLM) and 6α-OHP glucuronidation exhibits Michaelis-Menten kinetics (as opposed to the atypical kinetics observed for 21-OHP glucuronidation). The V_{max} value for 6α-OHP glucuronidation by HLM is comparable to those for zidovudine and morphine glucuronidation, commonly used xenobiotic 'probes' for hepatic UGT2B7 activity (Court 2005; Miners et al., 2006). Of the human UGTs, UGT2B7 is arguably the most important in terms of drug metabolism. For example, UGT2B7 contributes to the glucuronidation of opioids (Coffman et al., 1998; Court et al., 2003; Stone et al., 2003), non-steroidal anti-inflammatory agents (Jin et al., 1993), anti-cancer agents (Miners et al., 1997; Innocenti et al., 2001), lamotrigine (Rowland et al., 2006) and zidovudine (Barbier et al., 2000; Court et al., 2003).

The data reported here provide further insights into the stereo- and regio- selectivity of hydroxy-steroid glucuronidation by human UGTs. Of the compounds not glucuronidated by HLM, the hydroxyl groups of 6β- and 7α- OHAD and 6β- and 11β- are axially oriented in rings B/C, while the hydroxyl group of 17α- OHP is hindered by the pregnane 17β side-chain. Thus, glucuronidation in these positions is presumably not favored due to steric hindrance.

Consistent with a previous report (Turgeon et al., 2001), UGT 2B15 and 2B17 selectively glucuronidated TST. K_m values were comparable with both enzymes, although the V_{max} for the UGT2B17 catalyzed reaction was two orders of magnitude higher. However, since it is not possible to quantify the relative expression of these enzymes (in either HLM or HEK293 cells) comparison of intrinsic clearances

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(V_{max}/K_m) is not meaningful. UGT 2B15 and UGT 2B17 also exhibited the lowest K_m values (3 μM) with 16α-OHP. A comparison of the glucuronidation of 13 anabolic androgenic steroids by recombinant human UGTs (Kuuranne et al., 2003) and data from activity screening studies (Green et al., 1994; Beaulieu et al., 1996) suggest that UGT 2B15 and 2B17 preferentially glucuronidate 16α- and 17β- hydroxyl groups in ring D of androgens or pregnanes.

Also consistent with data presented here, UGT2B7 has previously been shown in this and other laboratories to glucuronidate numerous hydroxylated C18-, C19- and C21-steroids (Ritter et al., 1990; Jin et al., 1993 and 1997; Coffman et al., 1998; Turgeon et al., 2001; Kuuranne et al., 2003; Lepine et al., 2004). Apart from the substrates identified in the present study, UGT2B7 efficiently glucuronidates 3α-hydroxy androgens and pregnanes and 3, 4-catechol estrogens. Taken together, these data permit the development of structure-function relationships. It may be hypothesized that electrostatic interaction via the 20-keto function orientates the OH group of 3α-hydroxy-pregnanes for glucuronidation within the UGT2B7 active site (Fig 4A; 'normal' mode 1). (The presence of 16α- and 17β- hydroxy (and 17-keto) groups similarly orientate C18- and C19- 3-hydroxy-steroids within the UGT2B7 active site for glucuronidation at this position (Jin et al., 1997).) The preferential glucuronidation of 16α- and 21- OHP may be explained by binding to the UGT2B7 active site in the 'reverse' mode (ie. rotation through 180 along the x-axis), whereby an electrostatic interaction via the 3-keto function orientates these functional groups in a catalytically favorable orientation (Fig 4B). Similar considerations apply to the glucuronidation of ring B/C hydroxy-pregnanes (Fig 4C and 4D). Electrostatic interactions involving the the 3- and 20- keto groups orientate 6α-OHP and 11α-OHP for glucuronidation in the

'normal' and 'reverse' modes, respectively. (Alternatively, 6α- and 11α- OHP may be overlaid by rotation by 180 through the z-axis.). The normal and reverse binding modes illustrated in Figs 4C and 4D suggest that the equatorially oriented groups of 6β– and 12β– OHP might also be glucuronidated by UGT2B7, but these compounds were not available for investigation. Relationships between the two 'normal' binding modes (1 and 2) cannot be inferred from the present data.

UGT1A enzymes exhibit greater selectivity towards C19- and C21- hydroxy-steroids. As noted above, UGT 1A3, 1A4, 1A7, 1A8, 1A9 and 1A10 variably glucuronidated the compounds investigated here but, of the hepatically expressed enzymes, appreciable activity was observed only with the UGT1A4 catalyzed glucuronidation of 16α-OHP and the UGT1A9 catalyzed glucuronidation of 11α-OHP. Previous activity screening studies have reported that UGT 1A3, 1A4, 1A8, 1A9 and 1A10 have the capacity to glucuronidate hydroxylated C19 and C21 steroids (Green and Tephly 1996; Cheng et al., 1999; Kuuranne et al., 2003), although structure-function relationships with these enzymes were generally not investigated. Of interest was our observation that UGT1A10 glucuronidated most hydroxy-steroids screened for activity. Together with the activities identified for UGT 1A7 and 1A8, this indicates that many hydroxy-steroids may potentially be glucuronidated by enzymes present in the gastrointestinal tract.

In summary, comparative kinetic and inhibitor studies with HLM and a panel of recombinant human UGTs demonstrated that 6α- and 21- OHP may serve as substrate probes for human hepatic UGT2B7 activity in vitro. Of these compounds, 6α- OHP has advantages of higher activity and Michaelis-Menten kinetics (compared to the

atypical kinetics observed for 21-OHP glucuronidation). UGT2B7 was also identified as the enzyme responsible for the high affinity component of human liver microsomal 11α-OHP glucuronidation, whereas UGT2B15 and UGT2B17 were the major forms involved in human liver microsomal TST 17β-glucuronidation and the high affinity component of 16α-OHP glucuronidation. Activity of UGT1A subfamily enzymes towards the hepatically glucuronidated substrates was generally low. These data confirm the apparent preference of UGT2B subfamily enzymes in the glucuronidation of hydroxylated C19 and C21 steroids. Interestingly, however, UGT1A10, which is expressed only in the gastrointestinal tract, exhibited activity towards most of the glucuronidated substrates suggesting possible pre-hepatic first pass extraction of dietary hydroxy-steroids.

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FOOTNOTE

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Legends to Figures

Figure 1. Structures of the Δ^4-3-keto hydroxy-steroids.

Figure 2. Representative Eadie Hofstee plots for TST (panel A), 6α-OHP (panel B), 11α-OHP (panel C), 16α-OHP (panel D) and 21-OHP (panel E) glucuronidation by HLM (liver H13). Points show experimentally determined values while the curves are the computer generated curves of best fit.

Figure 3. Eadie Hofstee plots for TST, 6α-OHP, 11α-OHP, 16α-OHP and 21-OHP glucuronidation by the principal recombinant human UGT enzyme involved in the glucuronidation of each hydroxy-steroid (typically UGT 2B7 and 2B17). Points show experimentally determined values and the computer generated curves of best fit.

Figure 4. Proposed binding modes (panels A-D) of hydroxy-preganes in the UGT2B7 active site, showing sites of glucuronidation associated with each binding mode.

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Table 1: Incubation conditions for the kinetic characterization of hydroxy-steroid glucuronidation.

Substrate	**Enzyme Source**	**Protein Concentration** (mg/ml)	**Incubation time** (min)	**Substrate concentration range** (μM)
TST	HLM	0.3	15	1 – 25
	UGT2B15	1.0	90	1 – 25
	UGT2B17	0.1	20	1 – 25
6α-OHP	HLM	0.2	30	10 – 250
	UGT2B7	0.2	30	10 – 250
11α-OHP	HLM	0.05	10	1 – 100
	UGT1A9	0.2	15	5 – 200
	UGT2B7	0.5	20	1 – 50
16α-OHP	HLM	0.4	30	1 – 200
	UGT1A4	1.0	120	10 – 250
	UGT2B7	1.0	120	10 – 250
	UGT2B15	1.0	120	1 – 40
	UGT2B17	0.6	60	1 – 40
21-OHP	HLM	0.5	30	1 – 100
	UGT2B7	0.6	60	2.5 – 100

Abbreviations: TST, testosterone; 6α-OHP, 6α-hydroxyprogesterone; 11α-OHP, 11α-hydroxyprogesterone; 16α-OHP, 16α-hydroxyprogesterone; 21-OHP, 21-hydroxyprogesterone

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Table 2: Chromatographic conditions for the measurement of hydroxy-steroid glucuronides

Substrate	**Mobile Phase Gradient**		**Retention Times** (min)	
	Time (min)	**Composition**[a]		
TST	0 to 1 1 to 9.1 9.1 to 9.3 9.3 to 15	94%A, 6%D 35%A, 65%D 35%A, 65%D 94%A, 6%D	TST-Glu TST	5.2 9.5
6α-OHP	0 to 1 1 to 8.1 8.1 to 13	93%C, 7%D 45%C, 55%D 93%C, 7%D	6α-OHP-Glu 6α-OHP	6.1 8.8
11α-OHP	0 to 1 1 to 7.1 7.2 to 15	85%B, 15%D 50%B, 50%D 85%B, 15%D	11α-OHP-Glu 11α-OHP	4.2 7.7
16α-OHP	0 to 1 1 to 8.1 8.1 to 13	93%C, 7%D 45%C, 55%D 93%C, 7%D	16α-OHP-Glu 16α-OHP	5.7 8.3
21-OHP	0 to 1 1 to 6.5 6.5 to 10 10.1 to 15	85%B, 15%D 55%B, 45%D 55%B, 45%D 85%B, 15%D	21-OHP-Glu 21-OHP	4.7 9.5

[a] Mobile phase composition: A - water containing 5% acetonitrile; B - acetate buffer (20mM, pH 4.7) with 5% acetonitrile; C - 0.35mM acetic acid with 5% acetonitrile; D - acetonitrile

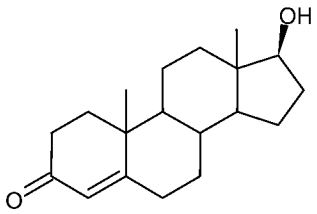
Table 3: Kinetic parameters for hydroxy-steroid glucuronidation by human liver microsomes (HLM) and expressed UGT enzymes

Hydroxy-steroid	Protein Source	Kinetic Model	K_{m1} or S_{50} (μM)	V_{max1} (pmol/min/mg)	K_{m2} (μM) or **n**	V_{max2} (pmol/min/mg)
	HLM	MM	5.6 ± 0.96[a]	196 ± 113[a]		
TST	UGT2B15	MM	5.7 ± 0.01[b]	4.5 ± 0.002[b]		
	UGT2B17	MM	3.8 ± 0.01[b]	508 ± 0.6[b]		
	HLM	MM	94 ± 22[a]	1828 ± 743[a]		
6α-OHP	UGT2B7	MM	55 ± 5[b]	269 ± 9[b]		
	HLM[c]	2MM	3.8 ± 1.8[a]	642 ± 278[a]	125 ± 172[a]	1293 ± 773[a]
11α-OHP	H7	MM	11 ± 0.4[b]	1622 ± 22[b]		
	UGT1A9	MM	93 ± 3.8[b]	1825 ± 38[b]		
	UGT2B7	MM	6.9 ± 0.2[b]	390 ± 3[b]		
	HLM	2MM	4.0 ± 1.5[a]	49 ± 29[a]	130 ± 103[a]	113 ± 46[a]
16α-OHP	UGT1A4	MM	73 ± 7[b]	1.5 ± 0.7[b]		
	UGT2B7	MM	211 ± 6[b]	8.6 ± 0.2[b]		
	UGT2B15	MM	3.0 ± 0.1[b]	1.8 ± 0.002[b]		
	UGT2B17	MM	3.0 ± 0.02[b]	14.7 ± 0.03[b]		
	HLM[d]	Hill	34 ± 12[a]	217± 54[a]	1.22 ± 0.14[a]	
21-OHP	H29	MM	72 ± 2[b]	113 ± 1[b]		
	UGT2B7	Hill	19 ± 1[b]	19 ± 0.5[b]	1.16 ± 0.06[b]	

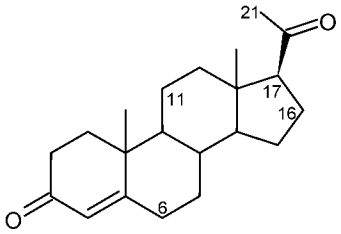
Abbreviations: HLM, human liver microsomes, MM, Michaelis-Menten equation; 2MM, 2-enzyme Michaelis-Menten equation; Hill, Hill equation.

[a] Mean ± SD (n = 4 or 5)
[b] Parameter ± parameter SE of fit
[c] All livers except H7
[d] All livers except H29

Figure 1

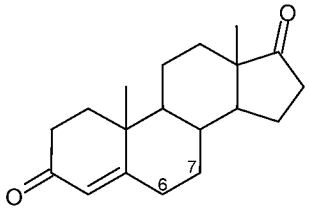


Testosterone



Progesterone (P) derivatives:

6α-OHP 16α-OHP
6β-OHP 17α-OHP
11α-OHP 21-OHP
11β-OHP



4-Androsten-3,17-dione (AD) derivatives:

6β-OHAD
7α-OHAD

Figure 2

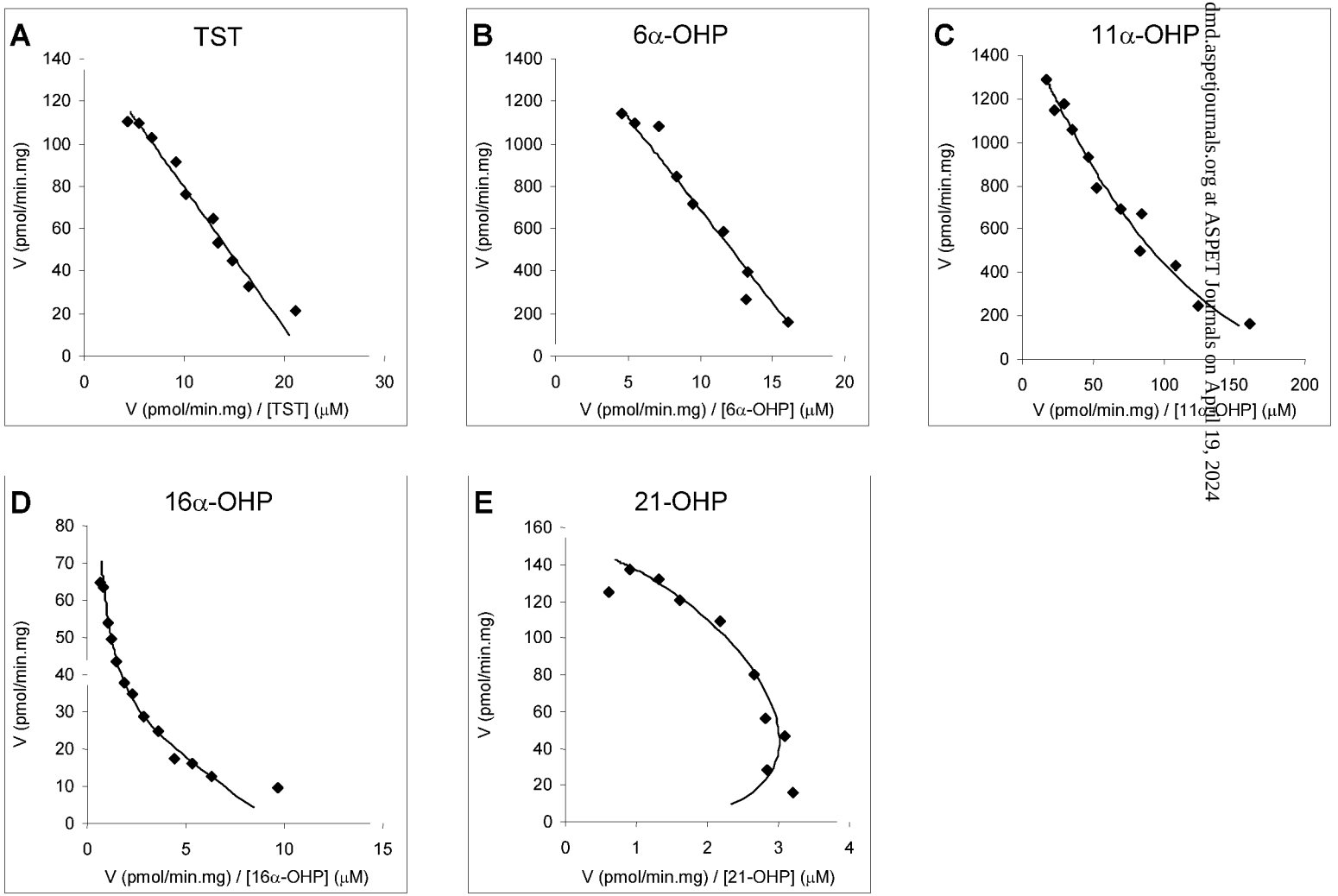
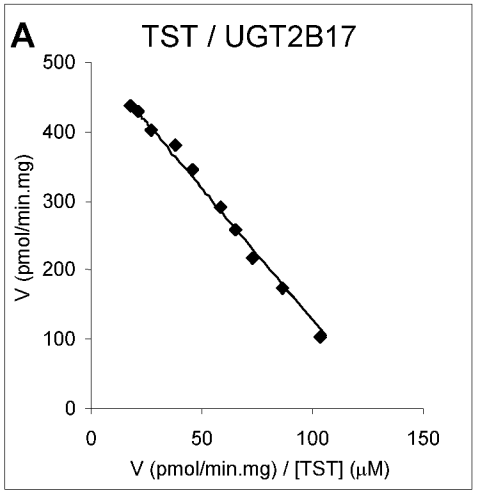
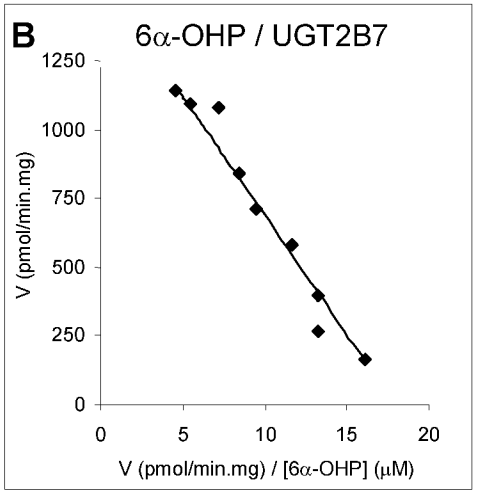
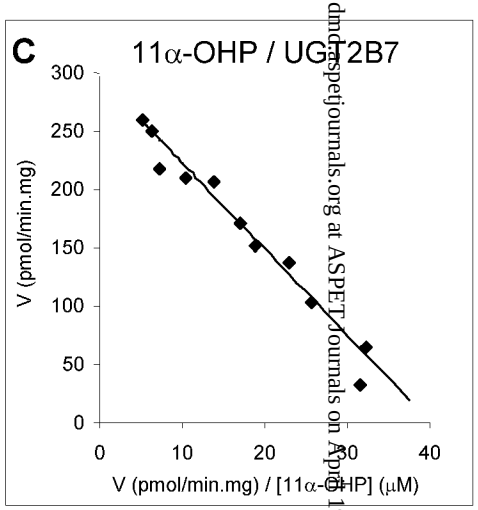
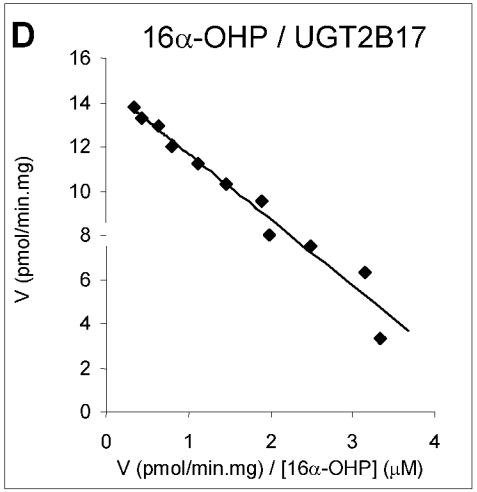


Figure 3









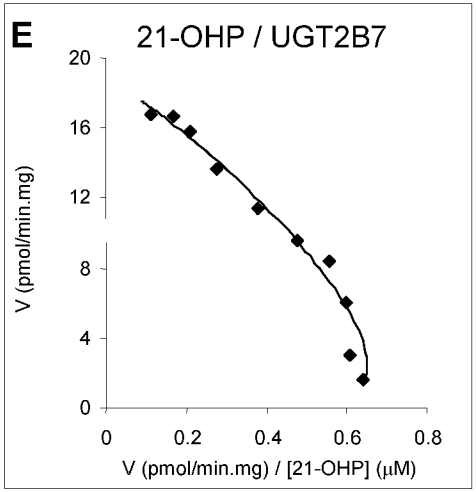
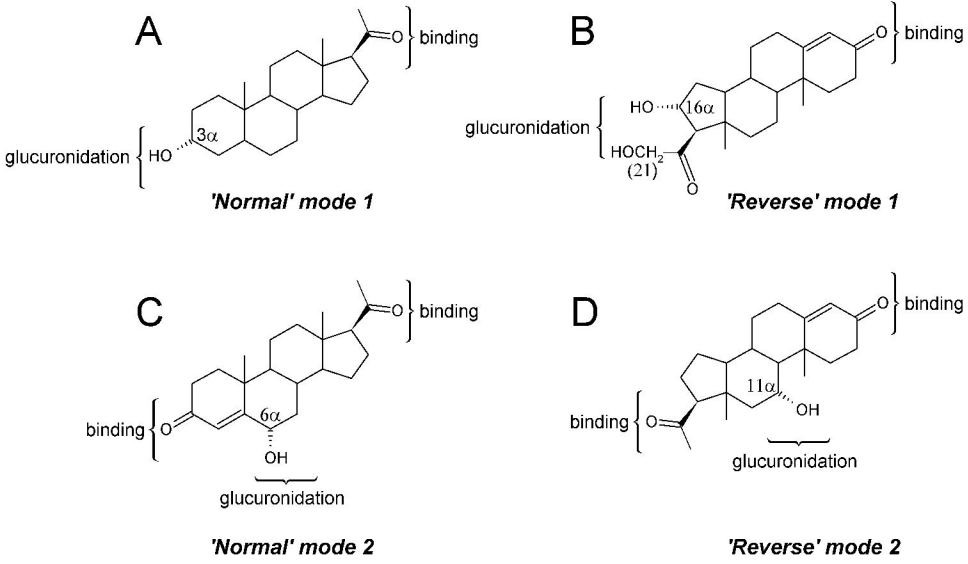


Figure 4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HO
16α
HOCH2
(21)
O
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C
O
binding
binding
O
6α
OH
glucuronidation
'Normal' mode 2
D
O
binding
binding
O
11α
OH
glucuronidation
'Reverse' mode 2