Correlation between Bilirubin Glucuronidation and Estradiol-3-Glucuronidation in the Presence of Model UDP-Glucuronosyltransferase 1A1 Substrates/Inhibitors

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Received June 28, 2010; accepted October 25, 2010

ABSTRACT:

Inhibition of UDP-glucuronosyltransferase (UGT) 1A1-catalyzed bilirubin glucuronidation by drug compounds may potentially be of clinical concern. However, in drug discovery and development settings, bilirubin is less than an ideal *in vitro* probe for assessing the potential of a chemical entity to inhibit bilirubin glucuronidation. In part, this is due to the propensity of bilirubin to photodegrade and to the instability of its metabolites. To this end, the utility of estradiol-3-glucuronidation as a surrogate *in vitro* predictor for interactions with bilirubin was evaluated. The glucuronidation kinetics of bilirubin and estradiol were carefully characterized with recombinant UGT1A1 expressed in human embryonic kidney 293 cells. Consistent with previous reports, estradiol-3-glucuronidation displayed sigmoidal kinetics, whereas bilirubin glucuronidation exhibited typical hyperbolic kinetics. The two compounds also mutually inhibited the metabolism of the other. Sixteen UGT1A1 substrates/inhibitors were evaluated as effectors of each reaction.

Fourteen compounds inhibited both bilirubin and estradiol glucuronidation. However, two compounds (ethinylestradiol and daidzein) exhibited mixed effects (concentration-dependent activation and inhibition) on estradiol-3-glucuronidation, whereas bilirubin glucuronidation was inhibited by both compounds. In addition, 7-ethyl-10-hydroxycamptothecin, a substrate of UGT1A1 (reported $K_m = 24 \mu\text{M}$) seemed to be a weak inhibitor of bilirubin glucuronidation ($\text{IC}_{50} = 356.4 \mu\text{M}$) but a partial inhibitor of estradiol-3-glucuronidation. The IC_{50} values of the inhibitors against estradiol-3-glucuronidation were strongly correlated with IC_{50} values against bilirubin glucuronidation, resulting in an R^2 value of 0.9604 (activator excluded) or 0.8287 (activator included). Thus, estradiol-3-glucuronidation can serve as a good surrogate for predicting inhibition of bilirubin glucuronidation with the caveat that occasionally compounds may demonstrate activation of estradiol-3-glucuronidation.

Introduction

UDP-glucuronosyltransferases (UGTs) catalyze the conjugation of glucuronic acid from the cofactor UDP-glucuronic acid (UDPGA) to lipophilic acceptor molecules (aglycones). This process increases the water solubility of the aglycones, facilitating their elimination (Radominska-Pandya et al., 1999). A wide variety of endobiotics, mutagens, drugs, and their metabolites are substrates of UGTs. In humans, the UGT enzymes have been divided into three subfamilies based on sequence homology: UGT1A, UGT2A, and UGT2B. Human UGT1A1 is an important glucuronidation enzyme. In addition to its ability to catalyze the glucuronidation of many xenobiotics including therapeutic drugs [e.g., ethinylestradiol, raltegravir, 7-ethyl-10-hydroxycamptothecin (SN-38), and buprenorphine] and mutagens (e.g., polycyclic aromatic hydrocarbons), UGT1A1 is the only physiologi-

cally relevant UGT capable of catalyzing bilirubin glucuronidation (Strassburg, 2008).

Bilirubin is a toxic endogenous compound formed through heme degradation. It has extremely low water solubility. UGT1A1-mediated glucuronidation is an essential step for efficient bilirubin elimination (Kadacol et al., 2000). The importance of this pathway of bilirubin elimination is further underscored in the development of unconjugated hyperbilirubinemia in individuals with Crigler-Najjar syndrome or Gilbert's syndrome wherein complete or partial loss of bilirubin glucuronidation capacity occurs because of genetic variants in the *UGT1A1* gene (Brierley and Burchell, 1993). To date, 113 single nucleotide polymorphisms have been identified within the *UGT1A1* gene (Alleles Nomenclature Home Page, <http://www.ugtalleles.ulaval.ca>). Note that *UGT1A1**28, a common polymorphism, is observed in approximately 40% of the Caucasian and African populations and accounts for most cases of Gilbert's syndrome in those populations (Beutler et al., 1998). In addition to genetic polymorphisms, xenobiotics inhibiting UGT1A1 (e.g., SN-38 and atazanavir) may also reduce bilirubin glucuronidation capacity, resulting in unconjugated hyperbilirubinemia (Rotger et al., 2005; Gupta et al.,

This work was supported in part by the National Institutes of Health National Institute of General Medical Sciences [Grant GM063215] (to T.S.T.); and in the form of an investigator-initiated grant from Bristol-Myers Squibb (to R.P.R.).

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

doi:10.1124/dmd.110.035030.

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; SN-38, 7-ethyl-10-hydroxycamptothecin; OH, hydroxy; HPLC, high-performance liquid chromatography; HEK, human embryonic kidney; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

2007). Therefore, in drug discovery and development settings, it is important to characterize the potential of a new chemical entity to inhibit UGT1A1 and by inference bilirubin glucuronidation.

To evaluate the potential inhibition of UGT1A1, ideally one would coinubate the compound of interest with a UGT1A1-specific probe substrate in human liver microsomes. Although bilirubin is highly specific for UGT1A1 and is the substrate of interest, it is less than an ideal UGT1A1 probe substrate for several reasons: 1) bilirubin and bilirubin glucuronides are chemically unstable; 2) authentic metabolite standards (bilirubin mono- and diglucuronides) are not commercially available; 3) bilirubin forms multiple metabolites; and 4) sequential metabolism occurs during the formation of bilirubin glucuronides (Miners et al., 2010). To date, the most widely used probe substrate for UGT1A1 has been estradiol (Court, 2005; Miners et al., 2010). In human liver microsomes, estradiol is conjugated by UGTs to form either the 3- or 17-glucuronide. Estradiol-3-glucuronidation is primarily catalyzed in the liver by UGT1A1, with some involvement of UGT1A3 (Lépine et al., 2004). Furthermore, formation of estradiol-3-glucuronide is highly correlated with bilirubin glucuronidation in human liver microsomes ($R^2 = 0.852$) (Zhang et al., 2007). In further support of the use of estradiol-3-glucuronidation as a measure of UGT1A1, beyond avoiding the technical challenges involved in measuring bilirubin glucuronidation, estradiol-3-glucuronide is commercially available and is easily measured. However, estradiol-3-glucuronidation has been reported to exhibit autoactivation (homotropic cooperativity) in human liver microsomes and recombinant UGT1A1 (Fisher et al., 2001; Udomuksorn et al., 2007). Atypical kinetic profiles, classified by homotropic and heterotropic cooperativity, have been increasingly reported with UGTs. Similar to the cytochromes P450, UGTs have been described to exhibit substrate-dependent autoactivation (Udomuksorn et al., 2007), substrate inhibition (Zhou et al., 2010b), and biphasic kinetics (Stone et al., 2003). Because of this propensity of the UGTs to exhibit atypical kinetic properties, a compound may activate the glucuronidation of one substrate but inhibit or have no effect on the glucuronidation of a second substrate catalyzed by the same UGT (Uchaipichat et al., 2008; Zhou et al., 2010b). In a recent study, we investigated the effect of tamoxifen on UGT1A4-catalyzed dihydrotestosterone, *trans*-androsterone, and lamotrigine glucuronidation and discovered that tamoxifen exhibited a concentration-dependent activation/inhibition of dihydrotestosterone and *trans*-androsterone glucuronidation, but only inhibited lamotrigine glucuronidation (Zhou et al., 2010b). Similar context-dependent heterotropic effects were also reported by Uchaipichat et al. (2008), who reported that 4-methylumbelliferone-activated UGT2B7 catalyzed 1-naphthol glucuronidation but inhibited zidovudine glucuronidation. Together, these results suggest the potential to make incorrect predictions of drug-drug interactions involving UGT enzymes when they are assessed with a single probe substrate.

Therefore, in present study we evaluated the appropriateness of using estradiol-3-glucuronidation as an *in vitro* predictor for interactions with bilirubin glucuronidation. To this end, the kinetic profiles for both estradiol-3-glucuronidation and bilirubin glucuronidation with recombinant UGT1A1 were carefully characterized, and the inhibition potentials of 16 UGT1A1 substrates/inhibitors on each process were compared.

Materials and Methods

Materials. Estradiol here refers to (17 β)-estra-1,3,5(10)-triene-3,17-diol and was purchased from Sigma-Aldrich (St. Louis, MO). Ritonavir, anthraflavic acid, 1-naphthol, ketoconazole, carvedilol, SN-38, 4-methylumbelliferone, 4'-OH-phenytoin, β -estradiol-3-glucuronide, levothyroxine, UDPGA, Trizma

base, Trizma HCl, D-saccharic acid 1,4-lactone, and alamethicin were also purchased from Sigma-Aldrich. Bilirubin IX α was purchased from Frontier Scientific (Logan, UT). Daidzein and riluzole were purchased from MP Bio-medicals (Salon, OH). Ethinylestradiol, raltegravir, niflumic acid, baicalein, and farnesol were purchased from Calbiochem (San Diego, CA), Toronto Research Chemicals Inc (North York, ON, Canada), Acros Organics (Fairlawn, NJ), Indofine Chemicals (Hillsborough, NJ), and TCI American (Portland, OR), respectively. MgCl₂ was purchased from Mallinckrodt (Hazelwood, MO). All other chemicals used were HPLC or reagent grade. Recombinant UGT1A1 was expressed in HEK293 cells (gift from Dr. Philip Lazarus, Penn State University, Hershey, PA) (Ren et al., 2000; Dellinger et al., 2006). Cell lysate, prepared by sonication of UGT1A1-HEK293 cells in 10 mM Tris buffer (pH = 7.4 at 37°C) containing 0.25 M sucrose for three 30-s bursts, each separated by 1-min cooling on ice, was added directly to the incubation as the enzyme source. The protein concentration in the cell lysates was determined with the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA).

Incubation Conditions to Characterize Kinetic Profiles. All incubations were conducted at 37°C in a final volume of 0.2 ml in 100 mM Tris-HCl buffer with 5 mM MgCl₂, 5 mM D-saccharic acid 1,4-lactone, 3 mM UDPGA, cell lysate (0.05 or 0.25 mg/ml), alamethicin (50 μ g/mg protein), and substrate. Estradiol and bilirubin were both dissolved in 100% dimethyl sulfoxide before being added to the incubation mixtures. The final dimethyl sulfoxide concentration in the incubations was 1%. Cell lysates were pretreated with alamethicin on ice for 30 min before reaction initiation. Preliminary experiments were conducted to ensure that all kinetic determinations were performed under linear conditions with respect to time and protein concentration. For estradiol-3-glucuronidation, incubations were carried out in the presence of 0.25 mg/ml protein for 30 min with 13 different estradiol concentrations (3–100 μ M). Reactions were terminated with 0.2 ml of cold methanol, followed by addition of 20 μ l of 1 μ g/ml *trans*-androsterone glucuronide as internal standard. The samples were then centrifuged at 13,000g for 5 min to remove the precipitated protein. Supernatant from each sample (5 μ l) were injected onto the HPLC system for quantification. Detailed incubation conditions for bilirubin glucuronidation were as described previously (Zhou et al., 2010a). Bilirubin incubations were conducted with 0.05 mg/ml protein for 5 min. These conditions assured that bilirubin consumption was less than 20% in all incubations. Bilirubin concentrations for kinetic profile characterization ranged from 0.05 to 2 μ M.

Interaction Studies. The effect of bilirubin on estradiol-3-glucuronidation (7.5, 15, and 30 μ M estradiol) was evaluated in the absence or presence of 0.04 to 1 μ M bilirubin. In addition, bilirubin glucuronidation (0.1, 0.2, and 0.4 μ M bilirubin) was studied in the absence or presence of 3 to 30 μ M estradiol. The effects of ritonavir, anthraflavic acid, 1-naphthol, ketoconazole, carvedilol, SN-38, 4-methylumbelliferone, 4'-OH-phenytoin, levothyroxine, daidzein, riluzole, ethinylestradiol, raltegravir, niflumic acid, baicalein, and farnesol were initially evaluated with 15 μ M estradiol or 0.2 μ M bilirubin. The concentrations of the effectors were originally selected on the basis of reported K_m or K_i values. The final concentrations (Table 1) of the effectors ensured that the percentage of remaining activity was more than and less than the 50% of control for calculation of IC₅₀ values. Because of the observation of atypical effects with daidzein and SN-38 in the initial studies, the effects of daidzein (0 and 3.125–50 μ M) and SN-38 (0 and 25–200 μ M) on estradiol-3-glucuronidation were further evaluated with multiple concentrations of estradiol (4–75 μ M).

Quantification of Estradiol-3-Glucuronide and Bilirubin Glucuronides. Methods for quantitation of bilirubin monoglucuronides and diglucuronide were as described previously (Zhou et al., 2010a). Estradiol-3-glucuronide was quantified either through an LC-MS method with an LC-10ADVP system (Shimadzu, Columbia, MD) or through a capillary LC-MS/MS method (for estradiol and bilirubin interaction experiments only) on a TSQ Quantum Discovery Max MS system (Thermo Fisher Scientific) coupled with an Agilent 1100 capillary HPLC system (Agilent Technologies, Santa Clara, CA). The mobile phase for both methods consisted of a 0.1% formic acid in water (A)-methanol (B) gradient and was delivered at a flow rate of 0.25 ml/min for the LC-MS method or 12 μ l/min for the capillary LC-MS/MS method. The HPLC column used for the LC-MS method was a Haisil Higgins C8 column (100 $\times$ 2.1 mm, 5 μ M; Higgins Analytical Inc., Mountain View, CA) and the

TABLE 1
Effects of 16 compounds on bilirubin glucuronidation and estradiol-3-glucuronidation

Name	Conc. ^a μM	IC ₅₀	
		Bilirubin Glucuronidation (Effect)	Estradiol-3-Glucuronidation (Effect)
Ritonavir	1.3–50	3.0 (inhibition)	1.7 (inhibition)
Antraflavic acid	2–40	3.6 (inhibition)	4.0 (inhibition)
Levothyroxine	0.6–10	4.9 (inhibition)	5.6 (inhibition)
Baicalein	0.5–10	7.0 (inhibition)	4.4 (inhibition)
Farnesol	13–60	47 (inhibition)	40 (inhibition)
4-Methylumbelliferone	28–550	100 (inhibition)	100 (inhibition)
4'-OH-phenytoin	15–300	120 (inhibition)	110 (inhibition)
Raltegravir	25–200	170 (inhibition)	190 (inhibition)
Riluzole	25–500	180 (inhibition)	160 (inhibition)
1-Naphthol	88–1700	240 (inhibition)	280 (inhibition)
Ketoconazole	6.3–100	27 (inhibition)	13 (inhibition)
Carvedilol	2.5–50	27 (inhibition)	12 (inhibition)
Niflumic acid	2–80	53 (inhibition)	13 (inhibition)
SN-38	6.3–400	360 (inhibition)	N.A. (partial inhibition)
Ethinylestradiol	2.5–50	21 (inhibition)	44 (activation and inhibition)
Daidzein	6.3–130	7.3 (inhibition)	120 (activation and inhibition)

^a Concentration ranges of the modifiers used for IC₅₀ determination.

column for the LC-MS/MS method was a BetaBasic 18 column (150 × 0.5 mm, 3 μm ; Thermo Fisher Scientific). Linear gradient elution programs were used for both methods. For the LC-MS method, the initial mobile phase contained 40% B, which was linearly increased to 95% B over 5 min and retained at 95% for an additional 5 min. The column was then reequilibrated at 40% of B for 4 min. The elution program for the LC-MS/MS method was initiated at 50% B, then increased to 95% B over 3 min, and then maintained at 95% B for an additional 9 min, followed by an 8-min column reequilibration at 50% B. The mass spectrometers were both operated in negative ion mode with an electrospray ionization interface. Quantitation was performed in single ion monitoring mode by monitoring $m/z = 447$ ($[\text{M} - \text{H}]^-$) for estradiol-3-glucuronide and $m/z = 465$ ($[\text{M} - \text{H}]^-$) for *trans*-androsterone glucuronide or in multiple reaction monitoring mode by monitoring a transition pair of m/z 465 → 287 for estradiol-3-glucuronide and 447 → 227 for *trans*-androsterone glucuronide. The MS parameters for the LC-MS method were as follows: nebulizing gas flow, 1.5 l/min; interface bias, −3.50 kV; interface current, −9.20 μA ; heating block temperature, 200°C; focus lens, +2.5 V; entrance lens, 50.0 V; radiofrequency gain, 5660; radiofrequency offset, 5210; prerod bias, +4.2 V; main rod bias, +3.5 V; aperture, −20.0 V; conversion dynode, +7.0 kV; detector voltage, 2.5 kV; CDL voltage, −25.0 kV; Q-array d.c., −35.0 V; and Q-array radiofrequency, +150.0 V. In the LC-MS/MS method, argon was used as the collision gas. The MS operating conditions were as follows for the LC-MS/MS method: spray voltage, 2800 V; sheath gas pressure, 10 mTorr; capillary temperature, 350°C; tube lens offset, −300; source collision-induced dissociation, 8 V; collision pressure, 2.2 mTorr; collision energy, 42 V; and quad MS/MS bias, 1.4.

Data Analysis. The Michaelis-Menten equation (eq. 1) and Hill equation (eq. 2) were fit to the kinetic data for bilirubin glucuronidation and estradiol-3-glucuronidation, respectively, via nonlinear regression with Sigma Plot 9.0 (Systat Software Inc., San Jose, CA). Selection of the appropriate model was determined by visual inspection of the Eadie-Hofstee plots, comparison of the second-order Akaike information criterion, and the residual sum of squares.

$$V_0 = \frac{V_{\max} \times [S]}{K_m + [S]} \quad (1)$$

$$V_0 = \frac{V_{\max} \times [S]^n}{S_{50}^n + [S]^n} \quad (2)$$

For the interaction experiments, rates were expressed as percentage of control. Mean control values were determined for each experiment. A value of ≥20% was used for classification of compounds that increased or decreased estradiol-3-glucuronidation or bilirubin glucuronidation as activators or inhibitors. IC₅₀ was determined by linear interpolation (Huber and Koella, 1993).

IC₅₀ values for bilirubin glucuronidation and for estradiol-3-glucuronidation were compared for correlation by linear regression and the coefficient of determination (R^2) was calculated.

A two-site model (Fig. 1A; eq. 3) (Kenworthy et al., 2001) was applied to describe the interaction of daidzein on estradiol-3-glucuronidation. In this model, the substrate binds cooperatively to two binding sites in the enzyme, and the modifier competes for both sites. The model also assumes that no interaction occurs between the two modifier molecules, but the binding of one modifier molecule to the enzyme induces cooperativity to the substrate similar to that of the substrate itself. Thus, at low substrate and modifier concentrations, enhancement of substrate binding induced by the modifier overcomes any competitive inhibition, resulting in activation. In addition, the modifier-enzyme-substrate complexes (AES and SEA in Fig. 1A) are productive, but the effective catalytic rate constants (k_p) of these complexes differ from that of the enzyme-substrate complex (ES) by a factor c , which determines the extent of the activation and the substrate concentration range in which activation may occur. The effect of SN-38 on estradiol-3-glucuronidation was best described with a three-site model (Fig. 1B; eq. 4) (Kenworthy et al., 2001). In this model, the substrate binds cooperatively to two binding sites in the enzyme, whereas the inhibitor binds to a distinct binding site. Thus, inhibition of the reaction occurs by decreasing $V_{\max}$ rather than by changing the binding constant of the substrate. In addition, the modifier does not affect the interaction between the two substrate molecules. Thus, the sigmoidicity of the reaction does not change with increasing modifier concentration. Also in this model, enzyme complexes containing the modifier are productive, resulting in incomplete inhibition even at a saturating concentration of inhibitor. Changes in the effective catalytic rate constant for enzyme complexes containing the inhibitor are reflected by the parameter c . The aforementioned multiple-site models assume rapid equilibrium (Segel, 1975). The kinetic parameter $V_{\max}$ equates to $2k_p[E]_t$, where $[E]_t$ is the total enzyme concentration and k_p is effective catalytic rate constant. K_s , K_a , and K_i are binding affinity constants. The parameter c reflects changes in k_p , whereas the parameters a and d reflect changes in binding affinity of the substrate.

$$V_0 = \frac{V_{\max} \cdot \left(\frac{[S]}{K_s} + \frac{[S]^2}{a \cdot K_s^2} + \frac{c \cdot [S] \cdot [A]}{a \cdot K_s \cdot K_a} \right)}{1 + \frac{2[S]}{K_s} + \frac{2[A]}{K_a} + \frac{[S]^2}{a \cdot K_s^2} + \frac{2 \cdot [S] \cdot [A]}{a \cdot K_s \cdot K_a} + \frac{[A]^2}{K_a^2}} \quad (3)$$

$$V_0 = \frac{V_{\max} \cdot \left(\frac{[S]}{K_s} + \frac{[S]^2}{a \cdot K_s^2} + \frac{c \cdot [S] \cdot [I]}{d \cdot K_s \cdot K_i} + \frac{c \cdot [S]^2 \cdot [I]}{a \cdot d \cdot K_s^2 \cdot K_i} \right)}{1 + \frac{2[S]}{K_s} + \frac{[I]}{K_i} + \frac{[S]^2}{a \cdot K_s^2} + \frac{2 \cdot [S] \cdot [I]}{d \cdot K_s \cdot K_i} + \frac{[S]^2 \cdot [I]}{a \cdot d \cdot K_s^2 \cdot K_i}} \quad (4)$$

FIG. 1. Multiple-site kinetic models. A, a kinetic model to explain the effect of daidzein on estradiol-3-glucuronidation (eq. 3). B, a kinetic model to explain the effect of SN-38 on estradiol-3-glucuronidation (eq. 4). k_p is the effective catalytic constant. K_s , K_a , and K_i are binding affinity constants. Constant c reflects the change in k_p , and constants a and d reflect changes in binding affinity. [The figure has been drawn on the basis of a figure in Kenworthy et al. (2001).]

Results

Kinetics of Bilirubin Glucuronidation and Estradiol-3-Glucuronidation. The kinetic properties of bilirubin glucuronidation and estradiol-3-glucuronidation were carefully characterized in a recombinant human UGT1A1 system. Bilirubin glucuronidation (total bilirubin glucuronide formation) displayed a classic hyperbolic kinetic profile (Fig. 2A), whereas estradiol-3-glucuronidation exhibited characteristics of autoactivation, with a sigmoidal kinetic profile (Fig. 2B). Kinetic data for bilirubin glucuronidation and estradiol-3-glucuronidation were fit to the Michaelis-Menten equation (eq. 1) and

the Hill equation (eq. 2), respectively. The derived K_m and V_{max} values for bilirubin glucuronidation were $0.20 \pm 0.02 \mu\text{M}$ and $165 \pm 4.3 \text{ pmol}/(\text{min per mg of protein})$ respectively. The derived S_{50} , V_{max} , and Hill coefficient (n) for estradiol-3-glucuronidation were $15.2 \pm 0.4 \mu\text{M}$, $115 \pm 1.7 \text{ pmol}/(\text{min per mg of protein})$ and 1.9 ± 0.1 .

Interaction between Bilirubin and Estradiol Glucuronidation.

Both bilirubin and estradiol inhibited the glucuronidation of the other compound (Fig. 3), and the effects were substrate concentration-dependent. As substrate concentration was increased, less inhibition was observed, as would be expected for competitive inhibition. However, because of the atypical kinetic properties of estradiol glucuronidation, a simple one-site competitive inhibition model did not adequately explain both sets of data. More data points are required to fit the results to a more complex model, and thus the results are reported in a qualitative manner (Fig. 3).

Effects of 16 Model UGT1A1 Substrates or Inhibitors on Bilirubin and Estradiol Glucuronidation. Fourteen of the compounds studied inhibited both bilirubin and estradiol glucuronidation (Table 1). Among these 14 compounds, ritonavir, anthraflavic acid, levothyroxine, riluzole, baicalein, farnesol, 4'-OH-phenytoin, 4-methylumbelliferone, raltegravir, and 1-naphthol exhibited very similar IC_{50} values (differences less than 2-fold) on both bilirubin glucuronidation and estradiol-3-glucuronidation (Table 1). Ketoconazole, carvedilol, and niflumic acid exhibited more disparity with respect to inhibition of the two reactions in that these compounds exhibited at least a 2-fold higher IC_{50} value against bilirubin glucuronidation than against estradiol-3-glucuronidation. SN-38 only weakly inhibited bilirubin glucuronidation ($\text{IC}_{50} = 356 \mu\text{M}$) and seemed to be a partial inhibitor of estradiol-3-glucuronidation. At even the highest SN-38 concentration, only 64% inhibition of estradiol-3-glucuronidation was noted. Two of the compounds examined, ethinylestradiol and daidzein, exhibited concentration-dependent activation/inhibition effects on estradiol-3-glucuronidation but were pure inhibitors of bilirubin glucuronidation. Daidzein and ethinylestradiol both activated estradiol-3-glucuronidation at low concentrations but inhibited the reaction at higher concentrations. The activation effect resulted in higher IC_{50} values (apparent IC_{50} , calculated without including the activation data points) on estradiol-3-glucuronidation than on bilirubin glucuronidation. Daidzein, which activated estradiol-3-glucuronidation to 156% of control, exhibited an ~ 16 -fold higher IC_{50} toward estradiol-3-glucuronidation than toward bilirubin glucuronidation. Ethinylestradiol, which modestly activates estradiol-3-glucuronidation (128% of control) exhibited an IC_{50} toward estradiol-3-glucuronidation that was ~ 2 -fold higher than the IC_{50} against bilirubin glucuronidation. The IC_{50} values for each inhibitor-substrate pair were plotted to assess whether a correlation existed in the inhibition of each substrate (bilirubin or estradiol) for these 16 compounds (Fig. 4). Excluding the data from the two heteroactivators (ethinylestradiol and daidzein), the R^2 value for the

FIG. 2. Kinetic plots (rate versus $[S]$) for bilirubin glucuronidation (A) and estradiol-3-glucuronidation (B). The bars indicate the range of triplicate measurements. The insets are Eadie-Hofstee plots for the same data. The Michaelis-Menten equation (eq. 1) was fit to the data for bilirubin glucuronidation. The sigmoidal equation (eq. 2) was fit to the data for estradiol-3-glucuronidation.

FIG. 3. Interactions between estradiol and bilirubin (rate percentage of control versus [modifier] plots). A, effect of estradiol on bilirubin glucuronidation. Symbols represent bilirubin concentration: 0.1 (●), 0.2 (○), and 0.4 (▼) μM . B, effect of bilirubin on estradiol-3-glucuronidation. Symbols represent estradiol concentrations: 7.5 (●), 15 (○), and 30 (▼) μM . Data points are means of duplicate measurements. Coefficients of variation are all within 10%.

correlation was 0.96 ($P < 0.0001$). If the two heteroactivators were included, the R^2 decreased to 0.83 ($P < 0.0001$).

Kinetics of Estradiol-3-Glucuronidation in the Presence of Daidzein. To more completely characterize the mixed effects (activation/inhibition) of daidzein on estradiol-3-glucuronidation, more detailed kinetics were assessed over a range of estradiol concentrations (4–75 μM). As depicted in Fig. 5A, the formation of estradiol-3-glucuronide was either activated or inhibited by daidzein, depending on both the estradiol concentration and the daidzein concentration. With increasing daidzein concentrations, the rate of estradiol-3-glucuronidation increased to a maximum at ~ 6.25 μM daidzein and then decreased with increasing daidzein concentrations. It is notable that greater activation of estradiol-3-glucuronidation by daidzein was observed at lower concentrations of estradiol, as is typically noted with heteroactivation (Hutzler et al., 2001; Kenworthy et al., 2001; Uchai-pichat et al., 2008). The most pronounced activation effect (392% of the control) was observed at the lowest substrate concentration (4 μM estradiol) in the presence of 6.25 μM daidzein. At 50 μM daidzein, slight inhibition of estradiol-3-glucuronidation was observed at estradiol concentrations greater than 15 μM . It is interesting to note that estradiol-3-glucuronidation displayed hyperbolic kinetics in the presence of daidzein, in contrast with the sigmoidal kinetic profile observed for this reaction when estradiol is incubated alone. The curvature noted in the Eadie-Hofstee plot for estradiol-3-glucuronidation in the presence of daidzein disappeared even at the lowest daidzein concentration (3.125 μM). The kinetic model presented in Fig. 1A (eq. 3) adequately described the kinetics of estradiol-3-glucuronidation

when coincubated with daidzein. The estimated kinetic parameters obtained by fitting the kinetic model (eq. 3) to the data are presented in Table 2, and the fit of data to eq. 3 is illustrated in Fig. 5B.

Kinetics of Estradiol-3-Glucuronidation in the Presence of SN-38. The partial inhibition of estradiol-3-glucuronidation during incubation with SN-38 noted at 15 μM estradiol led us to further investigate the interactions of SN-38 on the kinetics of estradiol-3-glucuronidation over a range of estradiol concentrations (4–75 μM). As depicted in Fig. 6A, partial inhibition by SN-38 was observed at all estradiol concentrations, and the extent of the maximum inhibition effect ($\sim 40\%$) was substrate concentration-independent. In addition, SN-38 did not alter the sigmoidicity observed with estradiol-3-glucuronidation by UGT1A1. Application of the Hill equation for analysis of the individual data sets at each inhibitor concentration demonstrated that the Hill coefficient n remained constant (coefficient of variation for the n values was 9%) over the full range of SN-38 concentrations. This result was further confirmed graphically by comparison of the Eadie-Hofstee plots of the individual data sets; comparable curvatures were noted (data not shown), again indicating that the sigmoidicity of estradiol-3-glucuronidation did not change in the presence of SN-38. A three-site kinetic model (Fig. 1B; eq. 4) adequately described the kinetics of estradiol-3-glucuronidation in the presence of SN-38. The kinetic parameters derived by simultaneous fitting of all kinetic data with the three-site model are shown in Table 2, and the fit is illustrated in Fig. 6B.

Discussion

It is important to assess the potential for new chemical entities to affect UGT1A1-catalyzed bilirubin glucuronidation. However, because of the technical challenges with the incubation and assay of bilirubin, it is desirable to develop alternate probes for this process. An alternate probe must be similar to bilirubin when assessed with a range of compounds that may be potential effectors of this process. Thus, the present study evaluated whether estradiol may be an appropriate probe for predicting interactions with UGT1A1-catalyzed bilirubin glucuronidation. Sixteen UGT1A1 substrates or inhibitors, which exhibit a wide range of reported K_m or K_i values and possess diverse chemical structures, were used as model effectors. Their effects on UGT1A1-catalyzed estradiol-3-glucuronidation and bilirubin glucuronidation were evaluated with a recombinant UGT1A1 system. Fourteen of the 16 compounds inhibited both reactions, with most exhibiting similar IC_{50} values toward both reactions. However, daidzein and ethinylestradiol resulted in concentration-dependent activation of estradiol-3-glucuronidation but not of bilirubin glucuronidation. Furthermore, SN-38, a UGT1A1 substrate, weakly inhibited bilirubin glucuronidation and exhibited only partial inhibition of estradiol-3-glucuronidation. Although differential effects on the two reactions were elicited by daidzein, ethinylestradiol, and SN-38, the IC_{50} values of all compounds (SN-38 was excluded because 50% of inhibition was not obtained.) toward the two reactions were highly

FIG. 4. Comparison of IC_{50} values for bilirubin glucuronidation and IC_{50} values for estradiol-3-glucuronidation. ●, modifiers that showed inhibition effect on both processes. ■, daidzein and estradiol, which showed heteroactivation on estradiol-3-glucuronidation. IC_{50} values for bilirubin glucuronidation and IC_{50} values for estradiol-3-glucuronidation were compared by linear regression, and the coefficient of determination (R^2) was calculated. Without the two heteroactivators (ethinylestradiol and daidzein), the R^2 for the correlation was 0.9702. If the two heteroactivators were included, the R^2 was 0.8435. The solid line represents the fit of all IC_{50} values to the linear equation.

FIG. 5. Effect of daidzein on estradiol-3-glucuronidation. A, rate percentage of control versus [modifier] plots. Symbols in A represent estradiol concentrations: 4 (●), 6 (○), 8 (▼), 10 (△), 15 (■), 20 (□), 25 (◆), 30 (◇), 50 (▲), and 75 (▽) μM. B, surface plot generated by fitting a two-site model (Fig. 1A; eq. 3) to the data.

correlated ($R^2 = 0.83$, $P < 0.0001$). This result suggests that, in general, estradiol is a suitable probe for predicting inhibition of bilirubin glucuronidation by recombinant UGT1A1. However, for high-throughput screening assays in which one substrate and one effector concentration are used, direct extrapolation may be tenuous because some effectors may activate estradiol-3-glucuronidation but inhibit bilirubin glucuronidation at certain concentrations. In addition, one must be aware of the contribution of UGT1A3 on estradiol-3-glucuronidation if human liver microsomes are used as the enzyme source. The correlation between estradiol-3-glucuronidation and bilirubin glucuronidation by human liver microsomes may be different from the results obtained in the present study with recombinant UGT1A1.

Consistent with previous reports (Fisher et al., 2001; Udomuksorn et al., 2007), in the present study estradiol-3-glucuronidation exhibited a sigmoidal kinetic profile. A widely accepted mechanism for sigmoidal kinetics involves cooperative binding of multiple substrate molecules to the enzyme (Segel, 1975; Korzekwa et al., 1998; Shou et al., 2001; Houston and Galetin, 2005). Thus, the sigmoidal kinetic profile exhibited by estradiol-3-glucuronidation suggested that two estradiol molecules may bind in the UGT1A1 active site simultaneously. In addition, the presence of atypical kinetics and, by inference, the presence of two binding regions within the active site of UGT1A1 may result in differential inhibition of estradiol-3-glucuronidation, depending on the inhibitor used.

Mutual inhibition was observed between estradiol and bilirubin. Bilirubin, unlike estradiol, displayed classic hyperbolic (Michaelis-Menten) kinetics. The observation of this type of kinetic profile implies that bilirubin either has one binding site or multiple identical and independent binding sites in UGT1A1 (Korzekwa et al., 1998). Nevertheless, the cross-inhibition between these two substrates together with the high correlation between bilirubin glucuronidation and estradiol-3-glucuronidation in the presence of model effectors suggests that the bilirubin binding region(s) and at least one of the estradiol binding regions are overlapping. In support of this hypothesis, Ciotti and Owens (1996) conducted site-directed mutagenesis studies and reported that bilirubin and ethinylestradiol bind to overlapping sites in UGT1A1. Structurally, ethinylestradiol only differs from estradiol by an ethinyl group at the 17-position of the steroidal scaffold. Ethinylestradiol-3-glucuronidation by UGT1A1 also resulted in a sigmoidal kinetic profile with an estimated S_{50} value of 10 μM (Soars et al., 2003), similar to the S_{50} obtained in the present study for estradiol. By inference, it is thus likely that estradiol and ethinylestradiol also share the same multiple binding sites in UGT1A1, at least one of which overlaps with the binding site(s) of bilirubin.

Among the 16 compounds screened in the present study, ethinylestradiol and daidzein exhibited a heteroactivation effect on estradiol-3-glucuronidation. In both cases, the activation effect became less pronounced with increasing concentrations of either compound,

TABLE 2

Kinetic parameters obtained by using multisite models to explain the effect of daidzein or SN-38 on estradiol-3-glucuronidation

Data are mean (S.E.).

Modifier	$V_{\max}$	K_{sub}	K_{mod}	a	c	d	Kinetic Model	R^2
	pmol/(min per mg of protein)	μM	μM					
Daidzein	190 (11)	81 (6.3)	6.3 (0.57)	0.11 (0.032)	2.6 (0.22)	N.A.	Eq. 3	0.96
SN-38	170 (5.9)	140 (21)	24 (5.4)	0.055 (0.018)	0.65 (0.028)	1.0 (0.11)	Eq. 4	0.99

N.A., not applicable, K_{sub} and K_{mod} refer to the binding affinity of the substrate and modifier to the enzyme.

FIG. 6. Effect of SN-38 on estradiol-3-glucuronidation. A, rate percentage of control versus [modifier] plots. Symbols represent estradiol concentrations: 4 (●), 6 (○), 8 (▼), 10 (△), 15 (■), 20 (□), 25 (◆), 30 (◇), 50 (▲), and 75 (▽) μM . B, surface plot generated by fitting a three-site model (Fig. 1B; eq. 4) to the data.

and at a high enough concentration, inhibition of estradiol-3-glucuronidation was observed. This type of concentration-dependent activation/inhibition effect has also been reported with other UGT isoforms (Uchaipichat et al., 2008; Zhou et al., 2010b). Daidzein, a major isoflavone component in soy, is a substrate of UGT1A1 (Nielsen and Williamson, 2007). Activation of UGT1A1 activity by daidzein in human liver microsomes has been noted previously by Pfeiffer et al. (2005). We confirmed a similar concentration-dependent activation/inhibition relationship of daidzein on estradiol-3-glucuronidation with recombinant UGT1A1. Also in agreement with their observations, we noted that the kinetic profile of estradiol-3-glucuronidation in the presence of daidzein displayed a hyperbolic (Michaelis-Menten) kinetic profile (Pfeiffer et al., 2005).

Multiple binding site models have been successfully used to explain various atypical (non-Michaelis-Menten) kinetic phenomena, including heteroactivation (Korzekwa et al., 1998; Shou et al., 2001; Tracy and Hummel, 2004; Houston and Galetin, 2005). In the present study, we applied a two-site kinetic model (Kenworthy et al., 2001) (Fig. 1A; eq. 3) to describe the heteroactivation effect by daidzein. As depicted in Fig. 1A, both estradiol and daidzein are proposed to bind to two unique sites within UGT1A1 and compete for binding at these sites. The predicted parameter a , which reflects changes in binding affinity of estradiol after one molecule of estradiol or daidzein binds to the enzyme, was substantially less than 1, indicating that binding of one molecule of either daidzein or estradiol to the enzyme facilitates the association of a second estradiol molecule to the other binding sites available within the enzyme active site. In addition, the kinetic parameter c , which is reflective of the formation of estradiol-3-glucuronide from the estradiol-enzyme-daidzein complex, was estimated to be 2.6, suggesting that formation of the estradiol-3-glucuronide was 2.6-fold faster from this complex than from the estradiol-enzyme complex.

Ethinylestradiol also activated estradiol-3-glucuronidation in the present study. However, the magnitude of the effect was much less than that observed with daidzein. Ethinylestradiol only slightly stimulated estradiol-3-glucuronidation (maximum of 128% of the control) at 15 μM estradiol. A similarly modest activation with these compounds was also observed by Mano et al. (2007) with recombinant UGT1A1 expressed in baculovirus-infected insect cells and by Wil-

liams et al. (2002) with a human liver microsomal preparation. Williams et al. (2002) also noted an increase in the magnitude of activation with decreasing substrate concentrations. With these results as a guide, we also evaluated the effect of ethinylestradiol on estradiol-3-glucuronidation with 7.5 μM (0.5 S_{50}) estradiol as substrate and observed a higher activation (up to 140% of the control).

SN-38, the active metabolite of irinotecan (an topoisomerase inhibitor used in cancer therapy), is primarily inactivated via glucuronidation and is a relatively specific UGT1A1 substrate (Mathijssen et al., 2001). SN-38 only partially inhibited estradiol-3-glucuronidation in a substrate concentration-independent manner. To model this type of inhibition, a three-site kinetic model (Houston and Galetin, 2005) was used wherein SN-38 binds to a distinct site separate from the two binding sites of estradiol (Fig. 1B; eq. 4). In this model, SN-38 did not alter the binding affinity of estradiol to the enzyme because the parameter d , reflective of changes in substrate affinity was estimated to be approximately unity. The inhibition by SN-38 was noted to result solely in a decrease in V_{max} . The parameter c was estimated to be 0.65, indicating that the effective catalytic rate constant for ESI and ESI₂ was 35% lower than that of the ES complex.

The reported K_m for SN-38 glucuronidation by recombinant UGT1A1 is $\sim 25 \mu\text{M}$, and it exhibited a hyperbolic kinetic profile (Hanioka et al., 2001). Of interest, SN-38 only weakly inhibited bilirubin glucuronidation. As described by the Cheng-Prusoff equation (Cheng and Prusoff, 1973), the IC_{50} values obtained at the apparent K_m should be twice the values of the K_i when the inhibition mechanism is competitive. However, the IC_{50} of SN-38 toward bilirubin glucuronidation was much higher than the predicted binding constant of SN-38 (Table 1) and the reported K_m for SN-38 glucuronidation. The discrepancy between the IC_{50} value of SN-38 and the reported K_m for SN-38 glucuronidation or its binding constant obtained in the present study suggest that the inhibition mechanism of SN-38 toward bilirubin glucuronidation was not competitive. It is likely that SN-38 also affects bilirubin glucuronidation through a distinct binding site, similar to that predicted for its interaction with estradiol-3-glucuronidation. Lack of inhibition of bilirubin glucuronidation by the UGT1A1 substrate has also been reported by Rios and Tephly (2002) with buprenorphine. These authors postulated that buprenorphine and bilirubin also bind to different domains within

UGT1A1 (Rios and Tephly, 2002). It is tempting to speculate that buprenorphine and SN-38 may share the same UGT1A1 binding domain, separate from the binding domain of both bilirubin and estradiol. However, studies of the interaction of buprenorphine and SN-38 would be required to test this hypothesis.

In summary, by evaluating the effects of 16 various UGT1A1 substrates/inhibitors on UGT1A1-catalyzed bilirubin and estradiol-3-glucuronidation, it was observed that estradiol-3-glucuronidation is an appropriate probe for predicting interactions with bilirubin glucuronidation because only minor disparities in correlation were noted. However, we have clearly identified compounds that are outliers. In addition, multisite kinetic analysis on the interaction of SN-38 on estradiol-3-glucuronidation suggested that these two UGT1A1 substrates bind to different binding sites in UGT1A1. These results suggest that multiple probes may still be necessary for predicting UGT1A1 inhibition or activation, as we recently found for UGT1A4 (Zhou et al., 2010b).

Acknowledgments

We thank Dr. Philip Lazarus at Penn State University for providing transfected HEK293 cells that express UGT1A1.

Authorship Contributions

Participated in research design: Zhou, Tracy, and Rimmel.

Conducted experiments: Zhou.

Contributed new reagents or analytic tools: Tracy and Rimmel.

Wrote or contributed to the writing of the manuscript: Zhou, Tracy, and Rimmel.

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