DIFFERENTIAL MODULATION OF UDP-GLUCURONOSYLTRANSFERASE 1A1 (UGT1A1)-CATALYZED ESTRADIOL-3-GLUCURONIDATION BY THE ADDITION OF UGT1A1 SUBSTRATES AND OTHER COMPOUNDS TO HUMAN LIVER MICROSONES

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ABSTRACT:

Previous results demonstrating homotropic activation of human UDP-glucuronosyltransferase (UGT) 1A1-catalyzed estradiol-3-glucuronidation led us to investigate the effects of 16 compounds on estradiol glucuronidation by human liver microsomes (HLM). In confirmation of previous work using alamethicin-treated HLM pooled from four livers, UGT1A1-catalyzed estradiol-3-glucuronidation demonstrated homotropic activation kinetics (S_{50} = 22 \mu M, Hill coefficient, n = 1.9) whereas estradiol-17-glucuronidation (catalyzed by other UGT enzymes) followed Michaelis-Menten kinetics (K_m = 7 \mu M). Modulatory effects of the following compounds were investigated: bilirubin, eight flavonoids, 17\alpha-ethynylestradiol (17\alpha-EE), estriol, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), anthraflavic acid, retinoic acid, morphine, and ibuprofen. Although the classic UGT1A1 substrate bilirubin was a weak competitive inhibitor of estradiol-3-glucuronidation, the estrogens and anthraflavic acid activated or inhibited estradiol-3-glucuronidation dependent on substrate and effector concentrations. For example, at substrate concentrations of 5 and 10 \mu M, estradiol-3-glucuronidation activity was stimulated by as much as 80% by low concentrations of 17\alpha-EE but was unaltered by flavanone. However, at higher substrate concentrations (25–100 \mu M) estradiol-3-glucuronidation was inhibited by about 55% by both compounds. Anthraflavic acid and PhIP were also stimulators of estradiol 3-glucuronidation at low substrate concentrations. The most potent inhibitor of estradiol 3-glucuronidation was the flavonoid tangeretin. The UGT2B7 substrates morphine and ibuprofen had no effect on estradiol 3-glucuronidation, whereas retinoic acid was slightly inhibitory. Estradiol-17-glucuronidation was inhibited by 17\alpha-EE, estriol, and naringenin but was not activated by any compound. This study demonstrates that the interactions of substrates and inhibitors at the active site of UGT1A1 are complex, yielding both activation and competitive inhibition kinetics.

Glucuronidation catalyzed by the UDP-glucuronosyltransferases (UGT) is a major pathway of metabolism of endogenous steroids, bile acids, drugs, carcinogens, and environmental pollutants (Teply and Green, 2000). Based on evolutionary divergence, two families of UGT enzymes have been identified, UGT1 and UGT2. The UGT1A gene is located on chromosome 2 and encodes for all members of the UGT1A subfamily by differential splicing of the gene product (Ritter et al., 1992). Within the UGT1A subfamily, the catalytic activity of UGT1A1 has been relatively well studied. Important physiological roles of UGT1A1 include glucuronidation of the toxic heme breakdown product bilirubin, as well as the glucuronidation of catechol estrogens, and flavonoids (Senafi et al., 1994). UGT1A1 also glucuronidates anthraquinones (Senafi et al., 1994), the oral contraceptive 17\alpha-ethynylestradiol (17\alpha-EE) (Ebner et al., 1993), and oripavine opioids such as buprenorphine (Senafi et al., 1994). In addition, UGT1A1-catalyzed estradiol-3-glucuronidation by microsomes from small intestine exceed rates found with liver microsomes (Fisher et al., 2000a). Functional polymorphisms in the UGT1A1 gene decrease bilirubin glucuronidation activity and depending on the nature of the polymorphism can lead to the toxicity associated with Gilbert syndrome, or the more severe Crigler-Najjar syndrome (Mackenzie et al., 2000).

Estradiol is glucuronidated at the 3-position by UGT1A1 (Senafi et al., 1994; Fisher et al., 2000a) and at the 17-position by several UGT enzymes, including UGT2B7 (Gall et al., 1999). The kinetics of estradiol-3-glucuronidation by UGT1A1 in human liver microsomes demonstrate homotropic activation (autoactivation kinetics) whereas estradiol-17-glucuronidation follows Michaelis-Menten kinetics (Fisher et al., 2000a,b). The mechanism of homotropic activation of UGT1A1-catalyzed estradiol-3-glucuronidation is unknown. One possibility is the existence of the enzyme in multimeric form, so that the binding of one substrate molecule to one subunit may increase the affinity of the other subunit(s) for another substrate molecule. A limited number of studies have provided experimental evidence to support the existence of UGT1A1 (Bruni and Chang, 1999) and UGT2B1 (Meech and Mackenzie, 1997) as multimeric enzymes.
Since UGT1A1-catalyzed estradiol-3-glucuronidation undergoes homotropic activation, the possibility also exists that UGT1A1 substrates or other compounds may act as heterotropic activators. Therefore, 16 structurally diverse compounds, including representatives of five distinct classes of compounds known to be UGT1A1 substrates were examined for their potential as activators and/or inhibitors of UGT1A1 activity.

### Table 1: Structures and modulatory effects of sixteen compounds on 3-glucuronidation of 5 μM estradiol

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Structure</th>
<th>Modulator range, μM</th>
<th>Stimulation/inhibition (5 μM estradiol)</th>
<th>IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLAVONE</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>5 to 100</td>
<td>Inhibition</td>
<td>n/a</td>
</tr>
<tr>
<td>FLAVANONE</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>5 to 100</td>
<td>No effect</td>
<td>n/a</td>
</tr>
<tr>
<td>NARINGENIN</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>10 to 100</td>
<td>No effect</td>
<td>n/a</td>
</tr>
<tr>
<td>CHRYSTIN</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>0.5 to 10</td>
<td>Inhibition</td>
<td>&lt;10</td>
</tr>
<tr>
<td>QUERCETIN</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>5 to 50</td>
<td>Inhibition</td>
<td>&gt;50</td>
</tr>
<tr>
<td>NOBILETIN</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>0.5 to 10</td>
<td>Inhibition</td>
<td>&gt;10</td>
</tr>
<tr>
<td>TANGERETIN</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>0.5 to 10</td>
<td>Inhibition</td>
<td>1</td>
</tr>
<tr>
<td>SILYMARIN</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>10 to 100</td>
<td>n/p</td>
<td></td>
</tr>
<tr>
<td>ANTHRAFLAVIC ACID</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>0.5 to 10</td>
<td>Stimulation at low concentration</td>
<td>n/a</td>
</tr>
<tr>
<td>BILIRUBIN</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>25 to 150</td>
<td>Inhibition</td>
<td>&lt;30</td>
</tr>
</tbody>
</table>

IC₅₀ values are given where appropriate and are based on the effects of four concentrations of modulator. n/p, not performed due to technical difficulties with the assay.
inhibitors of UGT1A1-catalyzed estradiol-3-glucuronidation over a wide range of effector and substrate concentrations. The known UGT1A1 substrates examined for modulatory effects on this activity were bilirubin, anthraflavic acid, 17α-EE (Tephy and Green, 2000), the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Malfatti and Felton, 2001), and the flavonoids naringenin and quercetin (Senafi et al., 1994). Four additional flavonoids, flavone, flavanone, chrysin, silymarin, and the two polymethoxyflavonoids, nobiletin and tangeretin, were also examined. The UGT2B7 substrates retinoic acid (Carrier et al., 2000), morphine (Coffman et al., 1997), and ibuprofen (Jin et al., 1993) were examined as examples of non-UGT1A1 substrates. Estriol (16α-hydroxy estradiol), which is not a substrate of UGT1A1 but is structurally related to 17α-EE, was included in the study to help determine whether the modulating effects of these compounds on estradiol-3-glucuronidation were unique to UGT1A1 substrates. In parallel, the effects of each of these compounds on estradiol-17-glucuronidation were also determined.

Materials and Methods

Chemicals. Unless stated all chemicals were purchased from the Sigma-Aldrich (St. Louis, MO). Tangeretin was obtained from Indofine Chemical Co. (Somerville, NJ). Nobiletin from orange oil was a gift from Dr. William Widmer, Florida Department of Citrus, Lakeland, FL. PhIP was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Morphine was obtained from Sigma/RBI (Natick, MA).

Liver Specimens. Human liver samples were obtained from the liver transplant unit at the Medical College of Wisconsin under a protocol approved by the Committee for the Conduct of Human Research. Microsomes from a mixture of four human liver samples (B, H, M, and P) were prepared by differential centrifugation as described previously (van der Hoeven and Coon, 1974) and mixed for use in these assays. The livers were chosen because they had average levels of estradiol-3-glucuronidation (UGT1A1) activities (Fisher et al., 2000a).

Assay for Modulation of Estradiol Glucuronidation Activity. The primary focus of the study was to investigate modulation of UGT1A1 activity using estradiol-3-glucuronidation as a probe. Preliminary experiments (data not shown) were performed to ensure estradiol glucuronidation experiments were carried out under initial rate conditions. Preliminary range finding experiments (data not shown) were also performed at an estradiol concentration of 25 μM (near its S_50 value, substrate concentration at half-maximal activity) to generate information on the potency of each compound as a modulator (at 5, 10, 30, 50, and 100 μM modulator) of estradiol-3-glucuronidation. Under these conditions, the 16 compounds either resulted in inhibition of estradiol-3-glucuronidation or had no effect. The results obtained in the preliminary studies were used to generate the appropriate ranges of modulator concentrations for subsequent experiments. Structures of each of the compounds are shown in Table 1.

The reaction conditions for assay of modulation of estradiol glucuronidation were as follows. Before each incubation, human liver microsomes (0.5 mg/ml),
Differential Modulation of UGT1A1 Activity In Vitro

0.1 M potassium phosphate buffer (pH 7.1), and alamethicin (25 µg/ml) were mixed and placed on ice for 15 min. This was followed by the addition of MgCl₂ (1 mM, final concentration), saccharolactone (5 mM, final concentration), and estradiol (25 µM for preliminary range-finding experiments, and 5, 10, 25, 50, and 100 µM in subsequent experiments) added in methanol (0.5% final concentration). Compounds tested for modulation of estradiol glucuronidation activity were added in methanol (0.5% v/v in the final incubation) except for bilirubin, morphine, retinoic acid, and anthracyclavice acid, which were added in dimethyl sulfoxide (0.5% v/v in the final incubation). As retinoic acid and bilirubin are light sensitive, these substrates were protected from light by aluminum foil throughout the experimental procedure. After a 3-min preincubation period in a shaking water bath (37°C), the reaction was started by the addition of UDPGA (5 mM, final concentration) to make a final incubation volume of 200 µl. After 30 min, the reaction was stopped by addition of ice-cold formic acid (25% v/v in buffer, 50 µl). α-Naphthylglucuronide (4 nmol) was added as internal standard, and the tubes were kept on ice for 30 min. The mixtures were centrifuged and supernatant transferred to vials for mass spectrometry analysis. The effects of 17α-EE (10 µM) on estradiol glucuronidation at 1.25, 2.5, 5, 10, 15, 20, 30, 50, 75, and 100 µM estradiol were also examined.

Analyses of glucuronide formation were performed on a Micromass platform LCZ system (Micromass Ltd., Manchester, UK) equipped with a Waters Alliance 2690 separations module and column oven (30°C), a 3-µm, 100 × 2 mm Prodigy ODS (3) high performance liquid chromatography column (Phenomenex, Torrance, CA) and a SecurityGuard column (Phenomenex). The mobile phase solution A was 10 mM ammonium acetate, and solution B was 90% acetonitrile/10% water/10% ammonium acetate. Initial conditions were 85% A/15% B pumped at 0.5 ml/min. A linear gradient from 15 to 31% B between 0 and 8 min was used, followed by 1 min at 100% B, and a re-equilibration at 15% B. Analytes were detected as their [M – H⁻] ions using negative ion electrospray ionization. The source block temperature was 125°C, desolvation temperature was 400°C, capillary voltage was –2.70 KV, and the cone voltage was –30V. The internal standard α-naphthylglucuronide, estradiol-3-glucuronide, and estradiol-17-glucuronide were detected by single ion monitoring at m/z 319, 447, and 447 and eluted at 5.6, 8.5 and 9 min, respectively. Standard curve correlation coefficients (r²) were ≥ 0.99.

Data Analysis. Duplicate values for rate of estradiol glucuronidation for each substrate concentration were fit to equations describing hyperbolic (Michaelis-Menten, eq. 1; Segel, 1975) or sigmoidal (Hill, eq. 2; Segel, 1975) relationships using WinNonlin software (Pharsight Corporation, Mountain View, CA). The best fit of the data to a relationship was determined according to established criteria (Ring et al., 1994).

\[
\nu = \frac{V_{\text{max}}[S]}{K_s + [S]} \quad (1)
\]

\[
\nu = \frac{V_{\text{max}}[S]}{[S] + S_0} \quad (2)
\]

Where enzyme kinetic data for estradiol glucuronidation best fit the Michaelis-Menten equation, Enzyme Kinetics/Pro (SynexChem, Fairfield, CA) and WinNonlin software were used to characterize the mode of inhibition (competitive, noncompetitive, mixed, or uncompetitive). Effects of each of the compounds at the lowest concentration of estradiol tested (5 µM) were determined, and if appropriate, IC₅₀ values were estimated. At this concentration of estradiol, compounds that increased or decreased estradiol glucuronidation by greater than 20% were respectively classed as stimulators or inhibitors (Table 1).

Results

Modulation of Estradiol-3-glucuronidation. In the absence of added modulator, enzyme kinetics consistent with homotropic activation were observed for estradiol-3-glucuronidation over a concentration range of 5 to 100 µM in each of 16 experiments. The mean V_{max} (± standard deviation) and S_{50} (± standard deviation) values for the 16 determinations were 433 ± 79 pmol/mg/min and 22 ± 5 µM. The mean Hill coefficient value (n), which gives an indication of the degree of sigmoidicity of the curve (n of 1 = Michaelis-Menten kinetics, no sigmoidicity) was 1.9 ± 0.6.

The structures of the 16 compounds examined are shown in Table 1. Figures 1 and 2 show the modulatory effect of each of the flavonoids and nonflavonoid compounds, respectively, over the indicated range of concentrations on estradiol-3-glucuronidation. Results are expressed as a percentage of control activity at estradiol concentrations of 5, 10, 25, 50, and 100 µM. Interestingly, several patterns of interaction between the modulators and UGT1A1-catalyzed estradiol-3-glucuronidation were observed. The rates of estradiol 3-glucuronidation at all estradiol concentrations examined were basically unaffected by the addition of increasing concentrations of the UGT2B7 substrates ibuprofen and morphine (Fig. 2). The pattern expected for a competitive inhibitor of estradiol 3-glucuronidation is a decrease in activity as the modulator concentration increases. In
addition for competitive inhibition, as substrate concentration increases the observed loss of glucuronide formation in the presence of inhibitor should decrease. Such patterns were observed for the effects of tangeretin (Fig. 1) and bilirubin (Fig. 2). However, as the kinetics of estradiol 3-glucuronide formation fitted the sigmoidal kinetic model best, and not hyperbolic kinetics described by the Michaelis-Menten equation, it was not appropriate to fit the inhibitory effects data to classic models of inhibition to generate $K_i$ values.

Naringenin inhibited estradiol 3-glucuronidation to a similar extent at all naringenin concentrations tested (Fig. 1) and therefore did not act as a competitive-type inhibitor. Flavone, quercetin (both Fig. 1), and retinoic acid (Fig. 2) were weak inhibitors of estradiol 3-glucuronidation at the modulator concentrations tested, however, it was difficult to determine from these results whether they were competitive-type inhibitors or not. For chrysin (5, 7-dihydroxyflavone), flavanone, nobiletin, silymarin (all Fig. 1), estril, and anthraflavic acid (both Fig. 2), the greatest inhibitory effect on estradiol 3-glucuronidation was at substrate concentrations above 25 $\mu$M. In addition, for chrysin, flavanone, nobiletin, silymarin (all Fig. 1), estril, and anthraflavic acid (Fig. 2), there seemed to be little effect, or possibly slight stimulation, at the lowest concentrations of estradiol examined (5 and 10 $\mu$M), principally at the lowest effector concentration. PhIP had a slight stimulatory effect at the lowest estradiol concentrations but had no inhibitory effects at any of the PhIP concentrations tested (Fig. 2).

Clear demonstration of heterotropic activation of UGT1A1 activity by 17$a$-EE was observed in two separate experiments designed to test for this phenomenon (Figs. 2 and 3). Figure 2 shows that at a substrate concentration of 5 $\mu$M, estradiol-3-glucuronidation was stimulated to 180% of the untreated value by 10 $\mu$M 17$a$-EE. However, at this substrate concentration, as the 17$a$-EE concentration increased, stimulation of estradiol-3-glucuronidation was attenuated so that by 50 $\mu$M 17$a$-EE, estradiol-3-glucuronide formation was 125% of the control activity and by 75 $\mu$M 17$a$-EE, approximately 20% inhibition was observed. An Eadie-Hofstee plot of this data (Fig. 3) clearly shows that increasing concentrations of 17$a$-EE (10 and 50 $\mu$M in this example) shift the shape of the line from a “curve” (observed in the absence of 17$a$-EE), which is typical of homotropic activation to a more linear relationship observed for Michaelis-Menten behavior. To more fully evaluate the effect of 17$a$-EE on estradiol glucuronidation, 10 $\mu$M 17$a$-EE was added to a wide range of estradiol concentrations, and its effects on estradiol 3-glucuronidation and estradiol 17-glucuronidation were examined (Fig. 4). The results demonstrate that 10 $\mu$M 17$a$-EE was shown to stimulate estradiol-3-glucuronidation by as much as 180% of control values at the low estradiol concentrations of 1.25 to 10 $\mu$M (below the $S_{50}$ value). This confirmed the observation of heterotropic activation at low 17$a$-EE and of substrate concentrations in the previously described experiment (Fig. 2) and that stimulation decreased with increasing substrate concentration. However, at higher effector concentrations, estradiol 3-glucuronida-
Differential Modulation of UGT1A1 Activity in Vitro

Fig. 4. Heterotropic activation of estradiol-3-glucuronidation and inhibition by 17α-EE dependence on substrate concentration.

With a wide range of estradiol concentrations, 17α-EE (10 μM) is seen to stimulate estradiol-3-glucuronidation at 1.25 to 10 μM estradiol and inhibit estradiol-3-glucuronidation at 15 to 100 μM, whereas estradiol-17-glucuronidation is inhibited at all estradiol concentrations tested.

Remember that isomerization formation was inhibited by 17α-EE (Fig. 2). Interestingly, as would be expected for competitive inhibition by 17α-EE, estradiol 17-glucuronidation was decreased by 10 μM 17α-EE at lower substrate concentrations (Fig. 4). In addition, as shown in Fig. 6, estradiol 17-glucuronidation was inhibited by 17α-EE in a concentration-dependent manner with the greatest inhibition occurring at the lowest substrate concentration.

Since estradiol-3-glucuronidation activity exhibited homotropic activation at low substrate concentration, and the compounds investigated appeared to differentially modulate the UGT1A1 enzyme at low versus high estradiol concentration, a classification of the effect of each compound was made at 5 μM estradiol (Table 1). Flavone, chrysin, quercetin, nobiletin, tangeretin, bilirubin, and retinoic acid were inhibitors of estradiol 3-glucuronidation at 5 μM. Heterotropic activation was observed for 17α-EE, PhIP, and anthraflavic acid. Flavanone, naringenin, ibuprofen, and estriol had no effect on estradiol 3-glucuronidation at a substrate concentration of 5 μM. Information on the effects of morphine and silymarin at 5 μM were not available due to technical difficulties with the assays. For silymarin, estradiol 3-glucuronidation was 150% of control activity at 25 μM estradiol and 10 μM silymarin (Fig. 1). There is no other data point above 120% of control activity in this graph (Fig. 1). It is therefore not appropriate to classify this compound as a stimulator (heterotropic activator), since the data at 5 μM estradiol was incomplete for this compound, and the apparent “stimulation” arising from a sporadic single data point does not seem to follow a trend with regard to estradiol concentration, as observed for 17α-EE, for example (described above and shown in Fig. 2).

Subtle differences in flavonoid structure had significant effects on the ability of the flavonoid to modulate estradiol 3-glucuronidation (Table 1). The ring nomenclature for flavonoids is shown on flavone. A hydroxyl group at position 3 is indicative of a flavonol (e.g., quercetin, Table 1), whereas saturation of the carbon atoms between position 2 and 3 is indicative of a flavone (Table 1). The most potent inhibitors of estradiol-3-glucuronidation at a concentration of 5 μM estradiol were the flavones, tangeretin (4',5,6,7,8-pentamethoxyflavone, IC50 = 1 μM) and chrysin (5,7-dihydroxyflavone, IC50 < 10 μM). The presence of a methoxy group at the 3' position appears to significantly reduce the inhibitory potency of flavones [e.g., nobiletin (3',4',5,6,7,8-hexamethoxyflavone, IC50 > 10 μM) compared with tangeretin]. Flavone, flavanone, silymarin, naringenin, and quercetin were considerably less potent than tangeretin or nobiletin in inhibiting estradiol 3-glucuronidation at a concentration of 5 μM estradiol (see Fig. 1 and Table 1).

Modulation of Estradiol-17-glucuronidation. Figures 5 and 6 show the effect of each of the flavonoids and nonflavonoid compounds, respectively over the indicated range of concentrations on estradiol 17-glucuronidation expressed as a percentage of control activity at each of the 5 estradiol concentrations examined. Hyperbolic Michaelis-Menten kinetics for the formation of estradiol 17-glucuronide were observed in each experiment yielding average Vmax and Km values (± standard deviations) for the 16 determinations of, respectively, 111 ± 26 pmols/mg/min and 7 ± 4 μM. 17α-EE was a competitive inhibitor of estradiol-17-glucuronidation (Ki = 20 μM) at the modulator concentrations tested. Estriol and naringenin were found to be noncompetitive inhibitors of estradiol 17-glucuronide and less potent inhibitors (Ki >50 μM) than 17α-EE. Interestingly, unlike the behavior observed for estradiol 3-glucuronidation, none of the examined compounds demonstrated a pattern of modulation consistent with stimulation of estradiol 17-glucuronidation (Figs. 5 and 6), although sporadic data points above 120% of control activity were observed in some cases (e.g., at 25 μM estradiol and 10 μM silymarin, and at 100 μM estradiol and 50 μM flavone).

Discussion

The results presented in this study demonstrate that, depending on effector and substrate concentrations, UGT1A1-catalyzed estradiol-3 glucuronidation activity is differentially sensitive to the effects of additional UGT1A1 substrates and other compounds. For those compounds (modulators) that stimulated estradiol 3-glucuronidation at estradiol concentrations below its S50 value, the greatest stimulation was observed at the lowest modulator concentrations (Figs. 1 and 2). This was confirmed by a separate, second experimental design using 17α-EE as modulator (Fig. 4). At higher concentrations of both modulator and substrate, no effect or inhibition was observed (Figs. 1 and 2). Although this is the first observation of heterotropic activation for a UGT enzyme, the in vitro observation of crossover from stimulation to inhibition as modulator concentration increases has also been observed for other enzymes involved in endo- and xenobiotic metabolism [e.g., cytochrome P450 (Ekins et al., 1998; Maenpaa et al., 1998; Stresser et al., 2000)]. The demonstration of heterotropic activation of UGT1A1-catalyzed estradiol-3-glucuronidation in the current study is consistent with the previous studies (Fisher et al., 2000a,b) indicating homotropic activation. Interestingly, homotropic activation kinetics for estradiol 3-glucuronidation activity have also been demonstrated using microsomes expressing recombinant UGT1A1 in our laboratory, indicating that this is a true behavior of the enzyme (unpublished observations).

Several compounds in the study were observed to be inhibitors of estradiol 3-glucuronidation by UGT1A1. Flavonoids were the most potent inhibitors with tangeretin being the most potent inhibitor of estradiol 3-glucuronidation. Unlike naringenin and the other hydroxylated flavonoids, which provide hydroxy groups for glucuronidation and are therefore substrates for UGT1A1, tangeretin has methoxy groups at various positions. It therefore appears that the most potent inhibitor determined in this study of UGT1A1-catalyzed estradiol-3 glucuronidation, tangeretin, is not a substrate of the enzyme.

The Hill equation (see “Data Analysis” under Materials and Meth-
The Hill coefficient value for UGT1A1-catalyzed estradiol 3-glucuronidation in the current set of experiments was on average 1.9. There is evidence for UGT1A1 existing as a tetramer (Peters and Jansen, 1986) and another UGT, UGT2B1 existing as a dimer (Meech and Mackenzie, 1997). Therefore, it is tempting to speculate that UGT1A1 behaves as a cooperative ligand-binding multisubunit enzyme in microsomes with regard to UGT1A1-catalyzed estradiol 3-glucuronidation, thus explaining the activation (homo- and heterotropic) of estradiol 3-glucuronidation.

There are alternative explanations for the cooperative behavior of UGT1A1 in human liver microsomes. One potential explanation is that allosteric effector sites exist on the UGT1A1 enzyme or that two substrate molecules occupy the UGT1A1 active site. For example, a kinetic model (Korzekwa et al., 1998) describing this binding and originally proposed to explain activation at low substrate concentrations and inhibition by effector at high substrate concentrations for CYP3A-catalyzed reactions (Ueng et al., 1997) adequately explains our data (results not shown). Therefore, should this proposal also hold...
for UGTs, these two sites may be within the UGT1A1 active site or on separate subunits, as described above. There is evidence of overlapping but distinct binding locations for 17α-EE and bilirubin in the UGT1A1 active site (Ciotti and Owens, 1996). However, there is no information to date on whether two estrogen-type molecules can simultaneously occupy the active site.

It may be argued that the observed heterotropic and homotropic activation of estradiol 3-glucuronidation is an artifact of the in vitro systems examined. The luminal location of UGT enzymes in the endoplasmic reticulum presents a particular problem for investigating UGT-catalyzed enzyme reactions. Sonication and detergents have been used to overcome this problem but ultimately result in disruption of the natural microenvironment of the enzymes. The alamethicin treatment (used in the current study) of microsomes is not thought to be as disruptive but does result in the free transit of substrate, cofactors, and products to and from the UGT enzyme (Fisher et al., 2000a). Thus the assay conditions used in the current studies appear to provide an optimal system for examining the behavior of the UGT enzymes. Furthermore, the lack of homotropic or heterotropic activation of estradiol 17-glucuronidation over the same effector and substrate concentration ranges demonstrates that activation is not a generalized artifact of the incubation conditions. It is therefore appropriate to conclude that the observations of homotropic and heterotropic activation for estradiol-3-glucuronidation are true behaviors of UGT1A1 at least in vitro. However, further studies will need to be performed to evaluate whether this in vitro phenomenon also occurs in vivo.

Observed $K_m/S_0$ values for UGT enzymes are often much higher than the plasma concentrations of substrates (Senafi et al., 1994). For example, plasma concentrations of estradiol are in the nanomolar range (Berrino et al., 2001), and the $S_0$ value for estradiol binding to UGT1A1 is 22 μM. If UGT1A1 behaves in vivo as it does in vitro, then at this low substrate concentration many compounds will be stimulators of estradiol 3-glucuronidation activity (Table 1). The findings of this study also raise an interesting point as to the ability of alamethicin-treated human liver microsomes to predict drug-drug interactions that result from the alteration of the catalytic activity of UGT1A1. It seems appropriate that for the most accurate prediction of drug-drug interactions, near-therapeutic concentrations of substrate (and inhibitor) should be used rather than the higher concentrations reflecting the substrate $K_m$ or $S_0$ value. This study design may provide a more relevant observation of enzyme behavior, as a compound predicted to be an inhibitor from an experiment using the substrate at $K_m$ concentrations may actually be a stimulator or have no effect at therapeutic concentrations in vivo.

Although it has not been possible to identify all of the enzymes that contribute to estradiol 17-glucuronidation in human liver microsomes, UGT2B7 is known to be a contributor (Gall et al., 1999). In the current study, three compounds were found to inhibit estradiol 17-glucuronidation. The competitive pattern of inhibition observed for estradiol 17-glucuronidation in human liver microsomes suggests that 17α-EE, estriol, and naringenin displace estrogen from the active site(s) of enzymes responsible for estradiol 17-glucuronidation. As already indicated, activation was not observed for this biotransformation. Thus it appears that Michaelis-Menten relationships between inhibitors and the substrate can be applied to the estradiol 17-glucuronidation results obtained in the current study.

In summary, evidence is provided for differential modulation, both activation and inhibition of estradiol-3-glucuronidation by other UGT1A1 substrates. For example, UGT1A1-catalyzed estradiol-3-glucuronidation is stimulated by 17α-EE at low substrate concentrations. In contrast, bilirubin is a weak competitive-type inhibitor. The most potent inhibitor of this activity at the lowest estradiol concentration tested is the polymethoxylflavone, tangeretin (IC$_{50}$ = 1 μM at 5 μM estradiol concentration). For compounds with similar structures to estrogens and anthraflavic acid, the stimulatory effects on estradiol 3-glucuronidation appear to be dominant over the inhibitory effects at low modulator concentrations, whereas at higher modulator concentrations, the inhibitory effects are dominant.

References


