 Estradiol 3-glucuronide is transported by the multidrug resistance-associated protein 2 but does not activate the allosteric site bound by estradiol 17-glucuronide

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ABSTRACT:
β-Estradiol 17-(β-D-glucuronide) (E217G) is a well known cholestatic agent and substrate of multidrug resistance-associated protein 2 (Mrp2), whereas β-estradiol 3-(β-D-glucuronide) (E23G) is a noncholestatic regioisomer of E17G with unknown transport properties. The purpose of this study was to compare and contrast the Mrp2-mediated transport of E17G and E23G. The full coding region of rat Mrp2 was cloned into the baculovirus genome, the recombinant baculovirus used to infect Sf9 cells, and ATP-dependent transport of 3H-E23G and 3H-E17G in Sf9 cell membranes was characterized. Mrp2 transported E23G into an osmotically sensitive space, requiring ATP, with S50 = 55.7 μM, Vmax = 326 pmol · mg⁻¹ · min⁻¹, and a Hill coefficient of 0.88. ATP-dependent Mrp2-mediated E17G transport was markedly stimulated at high E17G concentrations, consistent with positive cooperativity (Hill coefficient 1.5). E217G (5–125 μM) increased S50 but not Vmax for E23G transport, consistent with competitive inhibition. E23G (0.4–400 μM) completely, potently (IC50 = 14.2 μM), and competitively inhibited E17G transport, but E217G (0.01–250 μM) inhibited only 53% of E23G transport (IC50 = 33.4 μM). Estriol 16α-(β-D-glucuronide) potently and completely inhibited transport of E23G (IC50 = 2.23 μM), as did β-estradiol 3-sulfate 17-(β-D-glucuronide) (5–50 μM). In summary, E217G binds not only to an Mrp2 transport site, but also to an allosteric site that activates Mrp2 with positive cooperativity, thus activating its own transport and potentially that of other Mrp2 substrates, such as E23G. The noncholestatic E23G is an Mrp2 substrate and competes with E17G for transport, but does not activate the allosteric site.

During pregnancy, mean plasma levels of free and conjugated estrogens increase dramatically with gestational age (Levitz and Young, 1977). Additionally, 3-hydroxy glucuronidation of the steroid A ring appears to produce a unique terminal product in estriol conjugates (Levitz et al., 1984). We have postulated that increased levels of cholestatic estrogen glucuronides, such as β-estradiol 17-(β-D-glucuronide) (E17G) and estriol 16α-(β-D-glucuronide) (E16G), in pregnancy contribute to the decrease in bile secretory function observed in normal pregnancy. E17G is a prototype cholestatic estrogen D-ring glucuronide in the rat model, whereas β-estradiol 3-(β-D-glucuronide) (E3G) is a choleretic A-ring glucuronide (Meyers et al., 1981). The mechanistic basis for their differential effects on bile flow is unknown, but likely involves direct interactions with hepatic canalicular transporters. E217G is a well established Mrp2 probe substrate (Gerk and Vore, 2002), but it has not been determined whether E3G is an Mrp2 substrate. E23G inhibits 2,4-dinitrophenol-glutathione transport in rat canalicular membrane vesicles (Vore et al., 1996), and its biliary excretion in Mrp2-deficient Eisai hyperbilirubinemic rats is diminished (Takikawa et al., 1996), suggesting that E23G is an Mrp2 substrate.

Mrp2-mediated transport of E217G is required for its cholestatic activity, although the mechanism is not clear. E217G does not induce cholestasis in TR− rats deficient in Mrp2, even though very high concentrations of E217G were used, sufficient to achieve equal biliary concentrations of E17G in wild-type Wistar and TR− rats (Huang et al., 2000). These data imply that the interaction between Mrp2 and E17G is essential for cholestasis. We therefore questioned whether the inability of E3G to induce cholestasis is because it is not an Mrp2 substrate, or because its interaction with Mrp2 differs significantly from the interaction between Mrp2 and E217G.

E217G is transported by several ATP-binding cassette (ABC) transporters, including MDR1 (Huang et al., 1998), Mrp1 (Keppler et al., 1997), Mrp2 (Keppler et al., 1997), Mrp3 (Hirohashi et al., 1999), Mrp4 (Zelcer et al., 2003b), MRP7 (Chen et al., 2003), and ABCG2 (breast cancer resistance protein; or mitoxantrone resistance transporter) (Suzuki et al., 2003), but not Mrp6 (Belinsky et al., 2002). Additionally, E217G is transported by the rat organic anion-transporting polypeptides 1 to 4 (Hagenbuch and Meier, 2003). By contrast, little is known of the transport mechanisms for E23G. E23G is a poor inhibitor of Mrp1-mediated transport of leukotriene C4 and E217G (Lue et al., 1996), and is a low-affinity inhibitor of both MRP4 and

ABBREVIATIONS: E217G, β-estradiol 17-(β-D-glucuronide); ABC, ATP-binding cassette; E23G, β-estradiol 3-(β-D-glucuronide); E3SO17G, β-estradiol 3-sulfate 17-(β-D-glucuronide); E16G, estriol 16α-(β-D-glucuronide); EV, empty virus (i.e., lacking the Mrp2 gene); MDR, multidrug resistance transporter (P-glycoprotein); Mrp, multidrug resistance-associated protein; CI, confidence interval; Bsep, bile salt export pump.
MRP7, with IC$_{50}$ values near 100 μM (Chen et al., 2003; Zelcer et al., 2003b). E$_2$3G (100 μM) did not inhibit MDR1-mediated transport of E$_2$17G, suggesting that E$_2$3G is not an MDR1 substrate (Huang et al., 1998). E$_2$3G may be a substrate for rat organic anion-transporting polypeptide 1, since it inhibits E$_2$17G transport with a $K_i$ of 9.7 ± 0.7 μM (Kanai et al., 1996). Its substrate specificity for other transporters is unknown.

The purpose of this investigation was 1) to determine whether Mrp2 transports E$_2$3G, 2) to determine whether rat Mrp2-mediated E$_2$3G transport differs mechanistically from that of E$_2$17G, and 3) to obtain estimates of the kinetic parameters describing these interactions. The present research indicates that E$_2$3G, like E$_2$17G, is a high-affinity Mrp2 transport substrate. However, unlike E$_2$3G, E$_2$17G transport exhibits marked positive cooperativity, indicating that E$_2$17G, but not E$_2$3G, activates an allosteric site in Mrp2.

**Materials and Methods**

$^3$H-E$_2$17G (40–45 Ci:mmol) and $^3$H-E$_2$3G (53–57 Ci:mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Unlabeled estrogen conjugates (Fig. 1A) and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

The rat Mrp2 plasmid was obtained from Dr. Peter Meier, and the full coding region was inserted into the pFastBac1 vector (Invitrogen, Carlsbad, CA) with a nine-amino acid carboxy-terminal hemagglutinin tag and recombinated in DH10Bac Escherichia coli cells (Invitrogen) containing the baculovirus genome. A comparable carboxy-terminal 6×-histidine tag does not inhibit Mrp2 function (Hagmann et al., 2002). As a background control, the empty vector was also recombinated as above, but without an insert. The recombinant bacmids were amplified, sequenced (Elim Biopharmaceuticals, Hayward, CA) to confirm the presence (or absence) of Mrp2, and transfected using CellFectin (Invitrogen) into SJ9 insect cells. The supernatant containing the recombinant baculovirus was harvested, amplified, and titered by a viral plaque assay. SJ9 cells (5 × 10$^4$ cells) in suspension culture were infected (multiplicity of infection = 3), and 64 to 68 h later, cell membranes for transport experiments were harvested by layering on 38% sucrose and collecting the layer at the buffer-sucrose interface (Ito et al., 2001c). Membranes were vesiculated, snap frozen in liquid nitrogen, stored at −80°C, and designated as either Mrp2- or empty virus (EV)-infected cell membranes. Protein concentrations were determined by a modification of the method described using bovine serum albumin as a standard (Lowry et al., 1951).

Expression of rat Mrp2 was determined by Western blotting using 0.5 μg of sucrose-fractionated membrane protein. Proteins were denatured in the presence of sodium lauryl sulfate at 37°C for 30 min before loading onto an 8% Tris/glycine polyacrylamide Novex precast gel (Invitrogen), separated by standard electrophoresis, and transferred onto Polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH). Membranes were blocked using 5% nonfat milk at room temperature for 1 h, and binding of primary antibody [mouse anti-human MRP2 (M2 III-6); Alexis Biochemicals, San Diego, CA) and secondary antibody (sheep anti-mouse, horseradish peroxidase-conjugated; Amersham Biosciences Inc., Piscataway, NJ) was performed in 5% nonfat milk for room temperature for 1 h. Chemiluminescence detection was done using ECL-Plus (Amersham Biosciences Inc.) and exposure to Biomax MR film (Kodak, Rochester, NY)

Transport experiments were performed in a Tris-sucrose buffer (Ito et al., 2001c), containing 5 mM ATP or AMP, 10 mM MgCl$_2$, 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase, and unlabeled estrogen conjugates in either dimethyl sulfoxide (0.5%) or 10:4:1 Tris-sucrose buffer/propylene glycol/ethanol (2%) as vehicles. Preliminary studies showed that the choice of vehicle had no effect on transport. ATP-dependent transport of $^3$H-E$_2$17G or $^3$H-E$_2$3G into membrane vesicles (10 μg/20 μl) was measured in incubations at 37°C for 2 to 5 min, transport was stopped with 3.5 ml of ice-cold stop buffer (Ito et al., 2001c), and the mixture was quickly filtered onto Durapore 0.4-μm filters (Millipore Corporation, Bedford, MA). The filters were selected due to their minimal binding of E$_2$17G at low (90 nM) or high (100 μM) concentrations. The tubes and filters were rinsed as described (Boyer and Meier, 1990). $^3$H collected on the filters was detected by liquid scintillation counting using scintillation counting cocktail (Bio-Safe II; Research Products International Corp., Mt. Prospect, IL)

Transport corrected for that in the presence of AMP was termed ATP-dependent transport, whereas that corrected for background (EV) transport was termed Mrp2-mediated transport. Nonlinear regression was performed on saturation data by fitting the data to the Hill equation and weighting as indicated in figure legends. Other curves were unweighted for linear or nonlinear regression using Prism version 4 computer software (GraphPad Software Inc., San Diego, CA) for fitting as indicated in figure legends. Remaining data were analyzed by one-way analysis of variance (α = 0.05) followed by Dunnett’s multiple comparison test (Prism).

**Results**

The expected underglycosylated rat Mrp2 protein (<190 kDa) was detected in Mrp2-expressing SJ9 cell membrane vesicles by means of Western analysis (Fig. 1B). Mrp2 was undetectable in the EV membrane vesicles.

The basic transport characteristics for E$_2$3G and E$_2$17G are shown in Fig. 2. To ensure that transport properties and kinetic values were attributable to Mrp2, transport studies were carried out in sucrose-fractionated SJ9 plasma membranes, and transport in Mrp2-expressing membranes was corrected for that in membranes from cells transfected with the EV. Transport of both compounds was linear to at least
FIG. 2. Transport of E$_2$3G (A, C, and E) and E$_2$17G (B, D, and F) in sucrose-fractionated Sf9 membrane vesicles. Each data point represents mean ± S.D. from triplicate determinations. Filled or empty symbols represent data obtained from Mrp2-expressing or EV vesicles, respectively. A and B, E$_2$3G (30 nM) and E$_2$17G (77 nM) transport as a function of time. C and D, effect of sucrose (osmolarity) on E$_2$3G (30 nM) and E$_2$17G (77 nM) transport. Squares or circles represent data obtained in the presence of ATP or AMP, respectively. E, saturation of E$_2$3G transport. Nonlinear regression was performed fitting the data from two independent experiments (triplicate determinations) to the Hill equation, with a weighting factor of 1/y$^2$ (radiotracer concentrations 35–110 nM). F, data from three independent E$_2$17G saturation experiments, each determined in triplicate (radiotracer concentrations 43–96 nM). The data were fitted to the Hill equation (unweighted, Hill coefficient = 1.5).
5 min and was much greater in membrane vesicles expressing Mrp2 versus EV (Fig. 2, A and B). Similarly, transport of both compounds occurred into an osmotically sensitive space with a y-intercept close to zero, indicating that observed activity was mainly transport and binding was minimal (Fig. 2, C and D). Additionally, transport of both compounds was linear with respect to protein (data not shown).

Mrp2-mediated ATP-dependent transport of E2 3G (Fig. 2E) was fit to a single apparent saturable transport site with $S_{50} = 55.7$ (95% CI 44.2–70.1) μM and $V_{max} = 326$ (95% CI 286–366) pmol·mg$^{-1}$·min$^{-1}$, and a Hill coefficient of 0.88 (95% CI 0.85–0.91). Figure 2F shows the combined results of three independent saturation experiments with E2 17G. Fitting the data to simple linear (nonsaturable), one-site and two-site Michaelis-Menten equations and the Hill equations yielded the lowest Akaike information criteria (Prism 4.0) for the fit to the Hill equation, indicating that the fit to the Hill equation was best supported by the data. Due to the limited solubility of E2 17G, we were unable to fully saturate the transport to obtain unique estimates for $V_{max}$ and $K_m$; however, for Mrp2-mediated transport, the data were best fit to an unweighted Hill equation with a Hill slope of 1.5 ± 0.2. These data suggest positive cooperativity for E2 17G transport. Notably, E2 17G association with either Mrp2 or EV membrane vesicles in the absence of ATP was not influenced by E2 17G concentration (0.02–160 μM) or by any other agent used here. Also, ATP-dependent transport of E2 3G and E2 17G in EV membranes followed simple Michaelis-Menten kinetics (Hill slope = 1), with $K_m$ values of 221 (95% CI 100–490) μM and 56.2 (95% CI 33.2–79.3) μM, and $V_{max}$ values of 132 (95% CI 40–225) and 135 (95% CI 104–166) pmol·mg$^{-1}$·min$^{-1}$, respectively. Finally, E2 3G was more soluble than E2 17G under identical experimental conditions, avoiding solubility problems that occur at high E2 17G concentrations.

To better understand the structure-activity relationships among relevant estrogen glucuronides (Fig. 1A) and Mrp2, we compared E2 17G and E2 3G transport, and the effects of E3 16G, a weak cholestatic glucuronide (Meyers et al., 1981) and β-estradiol 3-sulfate 17-(β-D-glucuronide) (E2 3SO4 17G), a naturally occurring choleretic biliary estradiol metabolite (Meyers et al., 1980; Takikawa et al., 1996), on their transport. The results for E2 17G transport are shown in Fig. 3. Again, unique fits were not obtained for the E2 17G saturation data due to its limited solubility (Fig. 2F), but the data were best fit to the Hill equation with a Hill coefficient of 1.5. E2 3G (15 μM, Fig. 3A) shifted the entire E2 17G transport versus concentration curve (Hill coefficient fixed at 1.5) to the right, consistent with competitive inhibition for transport. E2 3G (IC$_{50}$ = 14.2 μM, 95% CI 7.8–25.7 μM, Fig. 3B) was a complete and high-affinity inhibitor of E2 17G transport. Additionally, E2 3SO4 17G (5–50 μM) potently and completely (50 μM) inhibited transport of E2 17G (Fig. 3C). Association or diffusion of E2 17G into either Mrp2 or EV membranes in the absence of ATP did not change with the concentrations tested. Concentrations of $^3$H-E2 17G above 250 μM increased the radioactivity collected on the filters in the absence of ATP, suggesting precipitation from solution. Furthermore, both MDR1-mediated E2 17G transport in S9 membranes (Huang et al., 1998) and E2 17G transport by EV

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**Fig. 3.** E2 17G transport and effects of estrogen glucuronides on E2 17G transport. Each data point represents mean ± S.D. of triplicate determinations from representative experiments. A, competitive displacement of E2 17G (111 nM) transport by unlabeled E2 3G (15 μM). B, inhibition of E2 17G (63 nM) transport by E2 3G. Data (unweighted) were fitted to a one-site competitive binding equation. C, inhibition of E2 17G (85 nM) transport by E2 3SO4 17G. Comparisons were made by Dunnett’s test following one-way analysis of variance. * indicates $p < 0.05$ versus 0 μM E2 3SO4 17G.
membranes in the present studies adhered to Michaelis-Menten kinetics. The data are therefore not consistent with nonspecific effects of E2 17G on the cell membrane.

Similar studies on E2 3G transport are shown in Fig. 4. E2 17G only partially inhibited (53%) E2 3G transport with an IC_{50} = 33.4 μM (95% CI 26.4–45.2 μM; Fig. 4A). In contrast, E3 16G was a complete and high-affinity inhibitor of E2 3G transport (IC_{50} = 2.23 μM, 95% CI 2.07–2.40 μM; Fig. 4B). Competitive inhibition studies were performed with E2 17G (5–125 μM) and E2 3SO_4 17G (5 μM) (Fig. 4, C and D), and the nonlinear regression fit to the Hill equation (Table 1). Neither E2 17G nor E2 3SO_4 17G had a significant effect on the V_{max} for E2 3G transport; as a result, V_{max} was shared for all the data sets. However, both E2 17G and E2 3SO_4 17G significantly increased the apparent S_{50} for E2 3G transport, indicative of competitive inhibition at the transport site. E2 3SO_4 17G and the highest E2 17G concentration also slightly increased the Hill coefficients closer to unity.

Discussion

The purpose of the present studies was to determine whether E2 3G is a substrate of Mrp2, to determine whether the noncholestatic E2 3G and the cholestatic E2 17G exhibited similar Mrp2-mediated transport properties, and to obtain estimates of the kinetic parameters for their transport. The present data clearly demonstrate that E2 3G is an excellent substrate for Mrp2, having an S_{50} of 55.7 μM and a V_{max} of 326 pmol/mg/min. Thus, although several permissive multidrug resistance transporters such as P-glycoprotein and the MRPs transport E2 17G, E2 3G is most likely not a substrate for MDR1 and MRP1 (Loe et al., 1996; Huang et al., 1998), as mentioned previously. This suggests a potentially important difference in the substrate specificities of MRP1 and Mrp2/MRP2. Thus, if E2 3G is transported by Mrp2 but not other MRPs or MDR1, E2 3G would be a more selective probe than E2 17G for MRP2-mediated transport. The higher water solubility of E2 3G also increases its experimental utility. The data also establish Mrp2 as a hepatic efflux mechanism for E2 3G, and are consistent with data demonstrating the choleretic activity of E2 3G (Meyers et al., 1980) and its biliary excretion in normal rats (Takikawa et al., 1996). Although E2 3G is not overtly toxic, its biliary excretion is important due to the complex pattern of interconversion among the estrogen metabolites (Levitz and Young, 1977; Levitz et al., 1984). During the preparation of this article, ethinyl-estradiol-3-glucuronide, a structural analog of E2 3G, was also shown to be transported by human MRP2 but not MRP1 (Chu et al., 2004), consistent with the present findings.

The present data also indicate that the interactions between E2 3G and E2 17G and Mrp2 are very distinct. The Hill coefficient for Mrp2-mediated E2 3G transport was slightly, but significantly less than unity (0.88). Although the significance of this negative cooperativity is not known, it could contribute to the somewhat higher S_{50} (56 μM) obtained for E2 3G transport versus its IC_{50} to inhibit E2 17G transport (14.2 μM). In contrast, E2 17G transport occurred with significant positive cooperativity (Hill coefficient of 1.5). Our preliminary data also indicate a Hill coefficient of 2 for human MRP2-mediated E2 17G transport (Gerk et al., 2003). Studies reporting K_{m} values of 3 to 8 μM for rat Mrp2 expressed in S9 cells (Ito et al., 2001b,c) or in HEK-293 cells (Cui et al., 1999) did not examine the range of E2 17G concentrations used here. At higher concentrations, E2 17G exhibited nonclassical transport kinetics, consistent with allosteric activation of its transport, as recently proposed for human MRP2 (Bodo et al., 2003; Gerk et al., 2003; Zelcer et al., 2003a).
cells clearly possess endogenous transporter(s) for both E2 17G and E2 3G, with \(K_m\) estimates of 56.2 and 221 \(\mu M\), respectively. It was necessary to correct for this endogenous transport activity, which became significant at high substrate concentrations, to obtain accurate estimates of the distinct kinetic parameters for Mrp2-mediated transport of E2 17G and E2 3G.

Evidence for two sites for E2 17G transport was also observed in the pattern of inhibition by other estrogen glucuronides. E2 3G and E2 3SO4 17G completely and potently inhibited E2 17G transport. Also, transport of E2 3G was potently inhibited by E3 16G (IC50 2.23 \(\mu M\)) and competitively inhibited by E2 3SO4 17G. Conversely, however, E2 17G did not completely inhibit E2 3G transport within the limits of E2 17G solubility. The data are consistent with a model in which E2 3G and E2 17G compete for binding to the Mrp2 transport site, as evidenced by the increased S0 for E2 3G transport (Fig. 4C; Table 1) and the rightward shift of E2 17G transport in the presence of E2 3G (Fig. 3A). E2 3G transport clearly does not demonstrate positive cooperativity, and therefore, E2 3G does not activate the allosteric site. The slight negative cooperativity of E2 3G transport (Hill coefficient 0.88) may reflect antagonism rather than activation of the allosteric site. The data in Fig. 4, A and C, support a model with an allosteric site having low affinity for E2 17G (\(K_m > 100 \mu M\)). Accordingly, low concentrations of unlabeled E2 17G competitively inhibit the transport of low concentrations of \(^1\text{H}-\text{E}_2\text{3G}\) or \(^1\text{H}-\text{E}_2\text{17G}\), but as E2 17G concentrations increase, activation at the allosteric site would occur concurrently with competition for the transport site. Such a model with concurrent activation and competition explains the positively cooperative transport of E2 17G and the inability of E2 17G to completely inhibit E2 3G transport, as well as the lack of further increase in S0 for E2 3G transport with increasing E2 17G concentrations (Table 1). The ability of E3 16G and E2 3SO4 17G to completely and potently inhibit transport also provides information on the nature of the transport site versus the allosteric site. The fact that E2 3SO4 17G inhibits completely indicates that sulfation of the A-ring of E2 17G abolished its ability to activate the allosteric site and implies that a phenolic A-ring is important for binding to this site. However, E3 16G, which like E2 17G is a glucuronide conjugate of the steroid D-ring with a phenolic A-ring, was also a potent and complete competitive inhibitor of E2 3G transport (IC50 2.2 \(\mu M\)); these data are also consistent with E2 16G having negligible activity at the allosteric site. These latter data indicate that the phenolic A-ring on a steroid glucuronide alone is not sufficient to activate the allosteric site; the greater hydrophilicity of E2 16G and/or the different stereochemistry of the glucuronic acid at the 16\(\alpha\)-OH of estriol versus the 17\(\beta\)-OH of estradiol are also likely critical factors that decrease activation of the allosteric site. Finally, these data indicate that the transport site is much more permissive than is the allosteric activation site.

The presence of more than one binding site on Mrp2 is consistent with findings with other ABC transporters that mediate efflux of xenobiotics and their conjugates. P-Glycoprotein has three proposed binding sites, including two transport sites and an allosteric site (Shapiro et al., 1999). The existence of this MDR1 allosteric site was recently confirmed (Maki et al., 2003), demonstrating that this allosteric site influences substrate translocation and its subsequent dissociation from MDR1. Separate binding sites for E2 17G and sulfinpyrazone have been postulated for human MRP2, based on substitutions at W1254F (Ito et al., 2001a). The W1254F MRP2 mutant retained E2 17G transport, but not leukotriene C4 or methotrexate transport. These authors concluded that each MRP2 substrate interacts with a unique, but overlapping set of contacts in a multipartite substrate-binding pocket. Also, Evers et al. (2000) postulated two MRP2 transport sites with positive cooperativity to explain the ability of sulfinpyrazone and indomethacin to stimulate glutathione transport. Whether the allosteric site described in the present studies represents another drug transport site or a third allosteric site is not clear. To date, E2 17G appears to be the only Mrp2 substrate that is able to activate its own transport.

The present data raise important questions regarding the physiological, pharmacological, and toxicological consequences of the ability of E2 17G to allosterically activate Mrp2 transport activity. The data suggest that binding of high, cholestatic concentrations of E2 17G to Mrp2 could lead to a marked stimulation of its transport into the canaliculus. Administration of E2 17G leads to endocytic retrieval of Mrp2 and Bsep from the canicular membrane, thus decreasing their ability to transport osmotically active solutes into bile and decreasing bile flow (Mottino et al., 2002; Crocenzi et al., 2003). A key question is whether E2 17G-induced activation of Mrp2 is causally related to E2 17G-induced internalization of Mrp2 and Bsep and cholestasis. Cell shrinkage has been shown to cause internalization of transporters and cholestasis (Haussinger et al., 2000), but at present, there is no evidence for a mechanistic link between Mrp2 activation and decreased cell volume sufficient to trigger such internalization. In the presence of Mrp2, E2 17G enhances activation of chloride channels and, in cells swollen with hypotonic media, causes cell shrinkage (Li and Weinman, 2002). Further studies are needed to determine whether E2 17G-induced activation of Mrp2 sufficient to cause Mrp2 and Bsep retrieval is preceded by shrinkage of hepatocytes.

The ability of estrogen glucuronides to serve as Mrp2 substrates and competitively inhibit Mrp2 transport may contribute to the decreased bile secretory function observed in normal pregnancy, and to intrahepatic cholestasis of pregnancy observed in some women. Although E1 16G has a lower cholestatic potency (29%) compared with E2 17G in animal models (Meyers et al., 1980), the concentrations of E1 16G (~100 nM) in human plasma at term of uncomplicated pregnancy are much greater than those of E2 17G (14 nM) (Levitz and Young, 1977; Numazawa et al., 1979). The present data show that E1 16G is a potent inhibitor of Mrp2, with an IC50 value near 2 \(\mu M\). Increased concentrations of E1 16G during normal pregnancy may reach levels in the hepatocyte sufficient to inhibit Mrp2. Further-
more, both E17G and E3G inhibit glutathione biliary excretion (Mottino et al., 2003). In intrahepatic cholestasis of pregnancy, a vicious cycle may occur in which the accumulation of cholestatic estrogen glucuronides progressively inhibits Mrp2 function by direct competitive inhibition as well as by triggering transporter retrieval, thus leading to further accumulation of cholestatic estrogen glucuronides and worsening of cholestasis.

In conclusion, these data provide direct evidence that E3G, like E17G, is transported by Mrp2, whereas E3SO417G and E16G are potent inhibitors of Mrp2 transport, consistent with their also being Mrp2 substrates. The interactions between Mrp2 and E17G are complex and require more than one distinct binding site. The data are consistent with a model in which E17G interacts with a transport site and an allosteric site that increases its own transport. E17G and E3G compete for binding to the transport site, but E3G does not activate the allosteric site. Mrp2 thus plays a major role in the biliary excretion of both cholestatic and noncholestatic estrogen glucuronides. Further studies are needed to identify the binding sites for E17G on Mrp2 and to determine whether there is a link between the binding of E17G to the allosteric site and its cholestatic activity.

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References

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