Estradiol 3-glucuronide is transported by the multidrug resistance-associated protein 2 (Mrp2) but does not activate the allosteric site bound by estradiol 17-glucuronide

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Running Title: Mrp2-Mediated Transport of Estrogen Glucuronides

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Non-Standard Abbreviations:

ABC       ATP-binding cassette
DNP-SG    2,4-dinitrophenol-S-glutathione
E23G      β-estradiol 3-(β-D-glucuronide)
E217G     β-estradiol 17-(β-D-glucuronide)
E23SO417G β-estradiol 3-sulfate 17-(β-D-glucuronide)
E316G     estriol 16α-(β-D-glucuronide)
EV        empty virus (ie., lacking the Mrp2 gene)
ICP       intrahepatic cholestasis of pregnancy
Mdr       multidrug resistance transporter (P-glycoprotein)
Mrp       multidrug resistance associated protein
Abstract:

β-Estradiol 17-(β-D-glucuronide) (E217G) is a well-known cholestatic agent and substrate of Mrp2, while β-estradiol 3-(β-D-glucuronide) (E23G) is a non-cholestatic regioisomer of E217G with unknown transport properties. The purpose of this study was to compare and contrast the Mrp2-mediated transport of E217G 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-E217G in Sf9 cell membranes characterized. Mrp2 transported E23G into an osmotically sensitive space, required ATP, with S50=55.7 µM, Vmax=326 pmol.mg⁻¹.min⁻¹, and a Hill coefficient of 0.88. ATP-dependent Mrp2-mediated E217G transport was markedly stimulated at high E217G 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 E217G 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 non-cholestatic E23G is an Mrp2 substrate and competes with E217G 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 metabolism, in contrast to the intermediary metabolism of other estriol conjugates (Levitz et al., 1984). We have postulated that increased levels of cholestatic estrogen glucuronides, such as β-Estradiol 17-(β-D-glucuronide) (E\textsubscript{217G}) and estriol 16α-(β-D-glucuronide) (E\textsubscript{316G}), in pregnancy contribute to the decrease in bile secretory function observed in normal pregnancy. E\textsubscript{217G} is a prototype cholestatic estrogen D-ring glucuronide in the rat model, whereas β-estradiol 3-(β-D-glucuronide) (E\textsubscript{23G}) 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. E\textsubscript{217G} is a well established Mrp2 probe substrate (Gerk and Vore, 2002) but it has not been determined whether E\textsubscript{23G} is an Mrp2 substrate. E\textsubscript{23G} inhibits 2,4-dinitrophenol-glutathione transport in rat canalicular membrane vesicles (Vore et al., 1996), and its biliary excretion in Mrp2-deficient EHBR rats is diminished (Takikawa et al., 1996), suggesting that E\textsubscript{23G} is an Mrp2 substrate.

Mrp2-mediated transport of E\textsubscript{217G} is required for its cholestatic activity, although the mechanism is not clear. E\textsubscript{217G} does not induce cholestasis in TR\textsuperscript{−} rats deficient in Mrp2, even though very high concentrations of E\textsubscript{217G} were used, sufficient to achieve equal biliary concentrations of E\textsubscript{217G} in wild type Wistar and TR\textsuperscript{−} rats (Huang et al, 2000). These data imply that the interaction between Mrp2 and E\textsubscript{217G} is essential for cholestasis. We therefore questioned whether the inability of E\textsubscript{23G} 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 (Oatp) 1-4 (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 (LTC4) and E217G (Loe et al., 1996), and is a low affinity inhibitor of both MRP4 and MRP7, with IC50 values near 100 µM (Chen et al., 2003; Zelcer et al., 2003b). E23G (100 µM) did not inhibit MDR1-mediated transport of E217G, suggesting that E23G is not an MDR1 substrate (Huang et al., 1998). E23G may be a substrate for rat Oatp1, since it inhibits E217G transport with a Ki of 9.7 ± 0.7 µM (Kanai et al., 1996). Its substrate specificity for other transporters is unknown.

The purpose of this investigation was to determine 1) whether Mrp2 transports E23G, 2) whether rat Mrp2-mediated E23G transport differs mechanistically from that of E217G and 3) to obtain estimates of the kinetic parameters describing these interactions. The present research indicates that E23G, like E217G, is a high affinity Mrp2 transport substrate. However, unlike E23G, E217G transport exhibits marked positive cooperativity, indicating that E217G, but not E23G, activates an allosteric site in Mrp2.
Methods:

$^3$H-E$_2$17G (40-45 Ci/mmol) and $^3$H-E$_2$3G (53-57 Ci/mmol) were obtained from Perkin-Elmer (Boston, MA). Unlabelled estrogen conjugates (Figure 1A) and all other reagents were obtained from Sigma (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 recombined in DH10Bac E. coli cells (Invitrogen) containing the baculovirus genome. A comparable carboxy-terminal 6x-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 Sf9 insect cells. The supernatant containing the recombinant baculovirus was harvested, amplified, and titered by a viral plaque assay. Sf9 cells (5 x10$^8$ cells) in suspension culture were infected (multiplicity of infection =3), and 64-68 hours 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 minutes before loading onto an 8% tris/glycine polyacrylamide Novex precast gel (Invitrogen), separated by standard electrophoresis and transferred onto Polynitran nitrocellulose membranes (Schleicher&Schuell, Keene, NH). Membranes were blocked using 5% nonfat milk at room temperature for one hour, and binding of primary antibody (mouse anti-human MRP2 (M2III-6), Alexis Biochemicals, San Diego) and secondary antibody (sheep anti-mouse, HPO-conjugated, Amersham, Piscataway, NJ) were performed in 5% nonfat milk at room temperature for one hour. Chemiluminescence detection was done using ECL-Plus (Amersham) 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 MgCl₂, 10 mM phosphocreatine, 100 µg/ml creatine phosphokinase, and unlabelled estrogen conjugates in either DMSO (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 ³H-E₂17G or ³H-E₂3G into membrane vesicles (10 µg/20 µl) was measured in incubations at 37°C for 2-5 minutes, transport stopped with 3.5 ml ice-cold stop buffer (Ito et al., 2001c) and the mixture quickly filtered onto Durapore 0.4 µm filters (Millipore, Bedford, MA). The filters were selected due to their minimal binding of E₂17G at low (90 nM) or high (100 µM) concentrations. The tubes and filters were rinsed as described (Boyer and Meier, 1990). ³H collected on the filters was detected by liquid scintillation counting using scintillation counting cocktail (Bio-Safe II, RPI, Mt. Prospect, IL).

Transport corrected for that in the presence of AMP was termed ATP-dependent transport, while 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, San Diego) for fitting as indicated in figure legends. Remaining data were analyzed by one-way ANOVA (\(\alpha=0.05\)) followed by Dunnett's multiple comparison test (Prism).
Results:

The expected underglycosylated rat Mrp2 protein (<190 kDa) was detected in Mrp2 expressing Sf9 cell membrane vesicles by means of Western analysis (Figure 1B). Mrp2 was undetectable in the EV membrane vesicles.

The basic transport characteristics for E23G and E217G are shown in Figure 2. In order to ensure that transport properties and kinetic values were attributable to Mrp2, transport studies were carried out in sucrose-fractionated Sf9 plasma membranes, and transport in Mrp2-expressing membranes was corrected for that in membranes from cells transfected with the empty vector (EV). Transport of both compounds was linear to at least 5 minutes and was much greater in membrane vesicles expressing Mrp2 vs EV (Figures 2A and 2B). 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 (Figures 2C and 2D). Additionally, transport of both compounds was linear with respect to protein (data not shown). Mrp2-mediated ATP-dependent transport of E23G (Figure 2E) was fit to a single apparent saturable transport site with \( S_{50} = 55.7 \) (95% CI 44.2-70.1) \( \mu \)M and \( V_{\text{max}} = 326 \) (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 E217G. Fitting the data to a simple linear (non-saturable), 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 E217G, we were unable to fully saturate the transport to obtain unique estimates for \( V_{\text{max}} \) 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 E217G transport. Notably, E217G association with either Mrp2 or EV membrane vesicles in the absence of ATP was not influenced by E217G concentration (0.02 to 160 µM) or by any other agent used here. Also, ATP-dependent transport of E23G and E217G in EV membranes followed simple Michaelis-Menten kinetics (Hill slope=1), with Km values of 221 (95%CI 100-490) µM and 56.2 (95%CI 33.2-79.3) µM and Vmax values of 132 (95%CI 40-225) and 135 (95%CI 104-166) pmol.mg⁻¹.min⁻¹, respectively. Finally, E23G was more soluble than E217G under identical experimental conditions, avoiding solubility problems that occur at high E217G concentrations.

To better understand the structure activity relationships amongst relevant estrogen glucuronides (Figure 1A) and Mrp2, we compared E217G and E23G transport, and the effects of estriol 16α-(β-D-glucuronide) (E316G), a weak cholestatic glucuronide (Meyers et al, 1981) and β-estradiol 3-sulfate 17-(β-D-glucuronide) (E23SO417G), a naturally occurring choleretic biliary estradiol metabolite (Meyers et al, 1980; Takikawa et al., 1996) on their transport. The results for E217G transport are shown in Figure 3. Again, unique fits were not obtained for the E217G saturation data due to its limited solubility (Figure 2F), but the data were best fit to the Hill equation with a Hill coefficient of 1.5. E23G (15µM, Figure 3A) shifted the entire E217G transport vs. concentration curve (Hill coefficient fixed at 1.5) to the right, consistent with competitive inhibition for transport. E23G (IC₅₀ = 14.2 µM, 95% CI 7.8 to 25.7 µM, Figure 3B) was a complete and high affinity inhibitor of E217G transport. Additionally, E23SO417G (5-50 µM) potently and completely (50 µM) inhibited transport of E217G (Figure 3C). Association
or diffusion of E217G into either Mrp2 or EV membranes in the absence of ATP did not change with the concentrations tested. Concentrations of 3H-E217G above 250 µM increased the radioactivity collected on the filters in the absence of ATP, suggesting precipitation from solution. Furthermore, both MDR1-mediated E217G transport in Sf9 membranes (Huang et al., 1998) and E217G transport by EV membranes in the present studies adhered to Michaelis-Menten kinetics. The data are therefore not consistent with nonspecific effects of E217G on the cell membrane.

Similar studies on E23G transport are shown in Figure 4. E217G only partially inhibited (53%) E23G transport with an IC50 = 33.4 µM (95% CI 26.4 to 45.2 µM, Figure 4A). In contrast, E316G was a complete and high affinity inhibitor of E23G transport (IC50 = 2.23 µM, 95%CI 2.07 to 2.40 µM, Figure 4B). Competitive inhibition studies were performed with E217G (5-125 µM) and E23SO417G (5 µM) (Figures 4C, 4D) and the nonlinear regression fit to the Hill equation (Table 1). Neither E217G nor E23SO417G had a significant effect on the Vmax for E23G transport; as a result, Vmax was shared for all the data sets. However, both E217G and E23SO417G significantly increased the apparent S50 for E23G transport, indicative of competitive inhibition at the transport site. E23SO417G and the highest E217G concentration also slightly increased the Hill coefficients closer to unity.
Discussion:

The purpose of the present studies was to determine if \( E_2 3G \) is a substrate of Mrp2, if the non-cholestatic \( E_2 3G \) and the cholestatic \( E_2 17G \) exhibited similar Mrp2-mediated transport properties, and to obtain estimates of the kinetic parameters for their transport. The present data clearly demonstrate that \( E_2 3G \) is an excellent substrate for Mrp2, having \( S_{50} \) of 55.7 µM and \( V_{\text{max}} \) of 326 pmol/mg/min. Thus, while several permissive multidrug resistance transporters such as P-glycoprotein and the MRPs transport \( E_2 17G \), \( E_2 3G \) 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 \( E_2 3G \) is transported by Mrp2 but not other MRPs or MDR1, \( E_2 3G \) would be a more selective probe than \( E_2 17G \) for MRP2-mediated transport. The higher water solubility of \( E_2 3G \) also increases its experimental utility. The data also establish Mrp2 as an hepatic efflux mechanism for \( E_2 3G \), and are consistent with data demonstrating the choleretic activity of \( E_2 3G \) (Meyers et al., 1980) and its biliary excretion in normal rats (Takikawa et al., 1996). While \( E_2 3G \) is not overtly toxic, its biliary excretion is important due to the complex pattern of interconversion amongst the estrogen metabolites (Levitz and Young, 1977; Levitz et al., 1984). During the preparation of this manuscript, ethinyl-estradiol-3-glucuronide, a structural analog of \( E_2 3G \) was also shown to be transported by human MRP2 but not MRP1, (Chu et al., 2004), consistent with present findings.

The present data also indicate that the interactions between \( E_2 3G \) and \( E_2 17G \) and Mrp2 are very distinct. The Hill coefficient for Mrp2-mediated \( E_2 3G \) transport was slightly, but significantly less than unity (0.88). While the significance of this negative
cooperativity is not known, it could contribute to the somewhat higher $S_{50}$ (56 µM) obtained for E$_2$3G transport vs its IC$_{50}$ to inhibit E$_2$17G transport (14.2 µM). In contrast, E$_2$17G transport occurred with significant positive cooperativity (Hill coefficient of 1.5). Our preliminary data also indicate a Hill coefficient of two for human MRP2-mediated E$_2$17G transport (Gerk et al., 2003). Studies reporting values of Km 3-8 µM for rat Mrp2 expressed in Sf9 cells (Ito et al., 2001b; Ito et al., 2001c) or in HEK-293 cells (Cui et al., 1999) did not examine the range of E$_2$17G concentrations used here. At higher concentrations, E$_2$17G exhibited nonclassical transport kinetics, consistent with allosteric activation of its transport, as recently proposed for human MRP2 (Bodo et al., 2003; Gerk et al., 2003; Zelcer et al., 2003a). Sf9 cells clearly possess endogenous transporter(s) for both E$_2$17G and E$_2$3G, with Km estimates of 56.2 and 221 µM, respectively. It was necessary to correct for this endogenous transport activity, which became significant at high substrate concentrations, to obtain accurate estimates of the distinct kinetic parameters for Mrp2-mediated transport of E$_2$17G and E$_2$3G.

Evidence for two sites for E$_2$17G transport was also observed in the pattern of inhibition by other estrogen glucuronides. E$_2$3G and E$_2$3SO$_4$17G completely and potently inhibited E$_2$17G transport. Also, transport of E$_2$3G was potently inhibited by E$_3$16G (IC$_{50}$ 2.23 µM) and competitively inhibited by E$_2$3SO$_4$17G. Conversely however, E$_2$17G did not completely inhibit E$_2$3G transport within the limits of E$_2$17G solubility. The data are consistent with a model in which E$_2$3G and E$_2$17G compete for binding to the Mrp2 transport site, as evidenced by the increased $S_{50}$ for E$_2$3G transport (Fig 4C and Table 1) and the rightward shift of E$_2$17G transport in the presence of E$_2$3G (Fig 3A). E$_2$3G transport clearly does not demonstrate positive cooperativity, and therefore
E₂³G does not activate the allosteric site. The slight negative cooperativity of E₂³G transport (Hill coefficient, 0.88) may reflect antagonism rather than activation of the allosteric site. The data in Figures 4A and 4C support a model with an allosteric site having low affinity for E₂¹⁷G (Km >100 µM). Accordingly, low concentrations of unlabeled E₂¹⁷G competitively inhibit the transport of low concentrations of ³H-E₂³G or ³H-E₂¹⁷G, but as E₂¹⁷G 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 E₂¹⁷G, and the inability of E₂¹⁷G to completely inhibit E₂³G transport, as well as the lack of further increase in S₅₀ for E₂³G transport with increasing E₂¹⁷G concentrations (Table 1). The ability of E₃¹⁶G and E₂³SO₄¹⁷G to completely and potently inhibit transport also provides information on the nature of the transport site vs the allosteric site. The fact that E₂³SO₄¹⁷G inhibits completely indicates that sulfation of the A ring of E₂¹⁷G abolished its ability to activate the allosteric site, and implies that a phenolic A-ring is important for binding to this site. However, E₃¹⁶G, which like E₂¹⁷G is a glucuronide conjugate of the steroid D-ring with a phenolic A ring, was also a potent and complete competitive inhibitor of E₂³G transport (IC₅₀ 2.2 µM); these data are also consistent with E₃¹⁶G 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 E₃¹⁶G and/or the different stereochemistry of the glucuronic acid at the 16α-OH of estriol vs the 17β–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 E217G and sulfinpyrazone have been postulated for human MRP2, based on substitutions at W1254 (Ito et al., 2001a). The W1254F MRP2 mutant retained E217G transport, but not LTC4 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. postulated two MRP2 transport sites with positive cooperativity to explain the ability of sulfinpyrazone and indomethacin to stimulate GSH transport (Evers et al., 2000). Whether the allosteric site described in the present studies represents another drug transport site or a third allosteric site is not clear. To date, E217G 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 E217G to allosterically activate Mrp2 transport activity. The data suggest that binding of high, cholestatic concentrations of E217G to Mrp2 could lead to a marked stimulation of its transport into the canaliculus. Administration of E217G 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 E$_{217}$G-induced activation of Mrp2 is causally related to E$_{217}$G-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, E$_{217}$G 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 if E$_{217}$G-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 (ICP) observed in some women. Although E$_{316}$G has a lower cholestatic potency (29%) compared to E$_{217}$G in animal models (Meyers et al., 1980), the concentrations of E$_{316}$G (~100 nM) in human plasma at term of uncomplicated pregnancy are much greater than those of E$_{217}$G (14 nM) (Levitz and Young, 1977; Numazawa et al., 1979). The present data show that E$_{316}$G is a potent inhibitor of Mrp2, with an IC$_{50}$ value near 2 µM. Increased concentrations of E$_{316}$G during normal pregnancy may reach levels in the hepatocyte sufficient to inhibit MRP2. Furthermore, both E$_{217}$G and E$_{23}$G inhibit glutathione biliary excretion (Mottino et al., 2003). In ICP, a vicious cycle may occur where 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 E23G, like E217G, is transported by Mrp2, while E23SO417G and E316G are potent inhibitors of Mrp2 transport, consistent with their also being Mrp2 substrates. The interactions between Mrp2 and E217G are complex and require more than one distinct binding site. The data are consistent with a model in which E217G interacts with a transport site and an allosteric site that increases its own transport. E217G and E23G compete for binding to the transport site, but E23G does not activate the allosteric site. Mrp2 thus plays a major role in the biliary excretion of both cholestatic and non-cholestatic estrogen glucuronides. Further studies are needed to identify the binding sites for E217G on Mrp2, and determine whether there is a link between the binding of E217G to the allosteric site and its cholestatic activity.
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References:


Footnotes

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Figure Legends

Figure 1. A, Structures of estrogen conjugates. B, Western Blot of Mrp2 expressed in insect cells. Abbreviations are as indicated in the text.

Figure 2. Transport of E$_2$3G (A, C, E) and E$_2$17G (B, D, F) in sucrose-fractionated Sf9 membrane vesicles. Each data point represents mean ± SD from triplicate determinations. Filled or empty symbols represent data obtained from Mrp2-expressing or EV vesicles, respectively. A and B, E$_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).

Figure 3. E$_2$17G transport and effects of estrogen glucuronides on E$_2$17G transport. Each data point represents mean ± SD of triplicate determinations from representative experiments. A, Competitive displacement of E$_2$17G (111 nM) transport by unlabelled E$_2$3G (15µM). B, Inhibition of E$_2$17G (63 nM) transport by E$_2$3G. Data (unweighted) were fitted to a one-site competitive binding equation. C, Inhibition of E$_2$17G (85 nM) transport by E$_2$3SO$_4$17G. Comparisons were
made by Dunnett’s test following one-way ANOVA, * indicates p<0.05 vs. 0 µM
E₂₃SO₄₁₇G.

Figure 4. E₂₃G transport and effects of estrogen glucuronides on E₂₃G transport. Each data point represents mean ± SD of triplicate determinations from representative experiments. **A** and **B**, Inhibition of E₂₃G (49 nM) transport by E₂₁₇G and E₃₁₆G. Data for **A** and **B** were fitted to a one-site competitive binding equation (unweighted). **C** and **D**, Competitive displacement of E₂₃G (35 nM) transport (squares) by unlabelled E₂₁₇G (**C**) and E₂₃SO₄₁₇G (5µM, **D**). Nonlinear regression was performed fitting the data to the Hill equation, with a weighting factor of 1/y² as discussed in the text.
Table 1. Kinetic analysis of Mrp2-mediated E$_2$3G transport in the presence of E$_2$17G or E$_2$3SO$_4$17G. Nonlinear regression results from data in Figure 4 C and D. Data were fitted as discussed in the text, with Vmax shared for all data sets. **Bold type** indicates 95%CI different from control.

<table>
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<th>Best-fit values</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Vmax (pmol.mg$^{-1}$.min$^{-1}$)</td>
<td>326</td>
<td>326</td>
<td>326</td>
<td>326</td>
<td>326</td>
</tr>
<tr>
<td>Hill coeff.</td>
<td>0.880</td>
<td>0.884</td>
<td>0.91</td>
<td><strong>0.981</strong></td>
<td><strong>0.932</strong></td>
</tr>
<tr>
<td>S$_{50}$ (µM)</td>
<td>55.7</td>
<td><strong>88.8</strong></td>
<td><strong>89.9</strong></td>
<td>82.4</td>
<td>99.5</td>
</tr>
</tbody>
</table>

95% Confidence Intervals

| Vmax (pmol.mg$^{-1}$.min$^{-1}$) | 286 to 366 | 286 to 366 | 286 to 366 | 286 to 366 | 286 to 366 |
| Hill coeff.               | 0.848 to 0.912 | 0.867 to 0.902 | 0.882 to 0.938 | **0.968 to 0.994** | **0.918 to 0.945** |
| S$_{50}$ (µM)             | 44.2 to 70.1 | **79.3 to 99.5** | **75.8 to 107** | 76.7 to 88.6 | 91.5 to 108 |
Figure 1.

A.

![Chemical structures of E₂3G, E₂17G, E₂3SO₄₁₇G, and E₃₁₆G](image)

B.

![Western blot showing expression of Rat Mrp2 and EV](image)

<185 kD>
Figure 2.

A. ATP-Dependent \(^{3}H\)-E23G Transport (pmol/mg) vs. Time (min)

B. ATP-Dependent \(^{3}H\)-E217G Transport (pmol/mg) vs. Time (min)

C. \(^{3}H\)-E23G Transport (fmol.mg\(^{-1}\).min\(^{-1}\)) vs. 1/Sucrose (1/M)

D. \(^{3}H\)-E217G Transport (fmol.mg\(^{-1}\).min\(^{-1}\)) vs. 1/Sucrose (1/M)

E. ATP-Dependent \(^{3}H\)-E23G Transport (pmol.mg\(^{-1}\).min\(^{-1}\)) vs. \(E_23G\) (µM)

F. ATP-Dependent \(^{3}H\)-E217G Transport (pmol.mg\(^{-1}\).min\(^{-1}\)) vs. \(E_217G\) (µM)
Figure 3.

A. 

B. 

C.
Figure 4.

A.

B.

C.

D.