0090-9556/09/3704-794–801$20.00
DRUG METABOLISM AND DISPOSITION
Vol. 37, No. 4

DMD 37:794–801, 2009
23895/3446830
Printed in U.S.A.

Multidrug Resistance Protein 2-Mediated Estradiol-17β-D-glucuronide Transport Potentiation: In Vitro-in Vivo Correlation and Species Specificity

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Received August 13, 2008; accepted December 31, 2008

ABSTRACT:

Multidrug resistance protein 2 (MRP2) is a multispecific organic anion transporter expressed at important pharmacological barriers, including the canalicular membrane of hepatocytes. At this location it is involved in the elimination of both endogenous and exogenous waste products, mostly as conjugates, to the bile. Estradiol-17β-D-glucuronide ($E_2$17βG), a widely studied endogenous substrate of MRP2, was shown earlier to recognize two binding sites of the transporter in vesicular transport assays. MRP2 modulators (substrates and nonsubstrates) potentiate the transport of $E_2$17βG by MRP2. We correlated data obtained from studies of different complexities and investigated the species-specific differences between rat and human MRP2-mediated transport. We used vesicular transport assays, sandwich-cultured primary hepatocytes, and in vivo biliary efflux in rats. Our results demonstrate that the rat Mrp2 transporter, unlike the human MRP2, transports $E_2$17βG according to Michaelis-Menten type kinetics. Nevertheless, in the presence of modulator drugs $E_2$17βG transport mediated by the rat transporter also shows cooperative kinetics as potentiation of $E_2$17βG transport was observed in the vesicular transport assay. We also demonstrated that the potentiation exists both in rat and in human hepatocytes and in vivo in rats.

MRP2 (ABCC2, cMOAT) is a member of the ATP-binding cassette transporter family. This efflux protein is expressed on the apical membrane of polarized cells and can be detected in many tissues, including the intestine, liver, and kidneys (for review, see Nies and Keppler et al., 2007). MRP2 has wide substrate specificity. Although it transports hydrophobic compounds in the presence of glutathione (Evers et al., 2000) its role in transporting anionic compounds and sulfate, glucuronide, and glutathione conjugates is considered more important (König et al., 1999). This transporter is also responsible for the biliary elimination of certain endogenous conjugates, such as leukotrienes and conjugated bilirubins. Inhibition of MRP2-mediated transport of these compounds by drug molecules may result in accumulation of toxic waste products in hepatocytes, precipitating hepatotoxicity and cholestasis (Bode et al., 2002; Zelcer et al., 2006). Indeed, naturally occurring mutations leading to deficiencies of human MRP2 (Dubin-Johnson syndrome) and rat Mrp2 (*TR*$^-$, Eisai hyperbilirubinemic rats) function (Buchler et al., 1996; Kartenbeck et al., 1996; Paulusma et al., 1997; Wada et al., 1998; Toh et al., 1999) caused increased blood levels of conjugated bilirubin metabolites. In addition, impaired canalicular excretion of a number of compounds in Mrp2-deficient rats has been shown (reviewed in Paulusma and Oude Elferink, 1997).

The transport of $E_2$17βG, an MRP2 substrate (Keppler et al., 1997), does not follow the classic Michaelis-Menten kinetics (Bodo et al., 2003; Zelcer et al., 2003; Zimmermann et al., 2008), rather the concentration dependence of transport follows sigmoid characteristics that can be explained by $E_2$17βG binding to two different sites on MRP2. Zelcer et al. (2003) referred to these sites as S (substrate) and M (modulator) and created four groups of MRP2 interactors based on their effect on $E_2$17βG transport. There are modulator compounds that are able to stimulate $E_2$17βG transport in a dose-dependent manner by competing with $E_2$17βG for the M site, which manifests as an increased transport of this substrate. $E_2$17βG is also a substrate of rat Mrp2, and this transporter mediates its biliary excretion (Morikawa et al., 2000). The transport kinetics of $E_2$17βG by rat Mrp2 is controversial as both hyperbolic and sigmoid transport profiles have been reported (reviewed in Borst et al., 2006a). Although most of these studies have been carried out in vesicular systems (reviewed in Borst et al., 2006a), the phenomenon has also been documented in cellular systems, albeit using substrates other than $E_2$17βG (Huisman et al., 2005; Zimmermann et al., 2008). However, the physiological rele-

This work was supported in part by the Hungarian Office for Research and Technology [Grants OTKA T 043141, GVOP-2004-3.3.2.-2004-04-0001/3.0, GVOP-3.1.1.-2004-05-0506/3.0]; Emberi Erőforrás Fejlesztés (Human Resources Development)-Munka 00034/2003; and the European Union [Grants FP6-NoE 005137, LSBH-CT-2006-518246, LSHB-CT-2006-037499].

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

doi:10.1124/dmd.108.023895.

ABBREVIATIONS: MRP2/Mrp2 (ABCC2, cMOAT), multidrug resistance protein 2; $E_2$17βG, estradiol-17β-D-glucuronide; Sf9, *Spodoptera frugiperda* ovarian; MOPS, 4-morpholinepropanesulfonic acid; HBSS, Hanks' balanced salt solution.

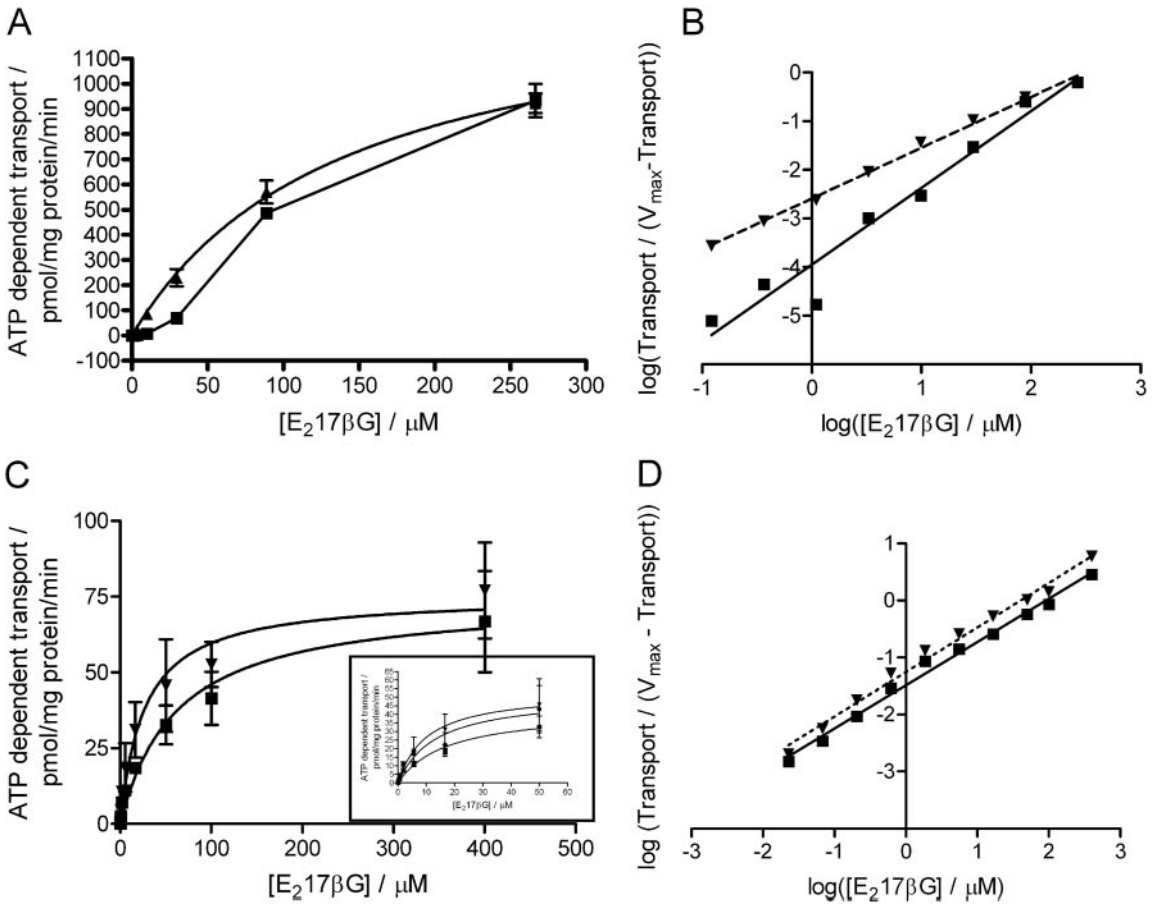

FIG. 1. Effect of indomethacin on the transport of $E_2$17βG by human MRP2 (A and B) and rat Mrp2 (C and D). For human MRP2 in the absence of 100 μM indomethacin (■), the estimated apparent half-maximal rate ($K_{0.5}$) was 150 μM, whereas in the presence of indomethacin (▲), the K_m is 139 μM and the V_{max} is 1413 pmol/mg protein/min. K_m and V_{max} values for rat Mrp2 in the absence of indomethacin (■) are 61.5 μM and 74.4 pmol/mg protein/min, respectively, whereas in the presence (▲) are 26.3 μM and 75.3 pmol/mg protein/min, respectively. Inset in C shows the effect of 50 μM indomethacin on $E_2$17βG transport by rat Mrp2 at lower substrate concentrations (K_m = 32.6 μM). Representative Hill plots are shown for human and rat protein in B and D, respectively, for transport in the absence (——) or in the presence (– – –) of 100 μM indomethacin.

vance of this phenomenon is unclear, as the modulator-induced potentiation of Mrp2-mediated $E_2$17βG transport, the most commonly studied probe substrate, has not been shown in vivo.

Another important question addressed is the difference in MRP2 substrate specificity between species. In preclinical studies mostly rodents are used to investigate the pharmacokinetics and toxicity of the compounds. Species specificity studies have been carried out for many MRP2 orthologs (Ninomiya et al., 2005, 2006; Zimmermann et al., 2008; Shilling et al., 2006). However, detailed studies that included membrane as well as cellular experimental systems have only been performed for the human and the mouse protein (Zimmermann et al., 2008).

In the present study, we investigated the correlation between data generated in experimental systems of different complexities: vesicular transport assay, sandwich-cultured rat and human hepatocytes, and in vivo rat studies. The second aim was application of the vesicular system and the sandwich-cultured hepatocytes to reveal differences between transporter orthologs of human and rat origin.

Materials and Methods

Materials. [^{3}H]Estradiol-17β-D-glucuronide ($E_2$17βG) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Recombinant baculovirus encoding wild-type human MRP2 was a kind gift from Balázs Sarkadi and András Váradi (Institute of Enzymology, Budapest, Hungary). Recombinant baculovirus harboring the rat Mrp2 cDNA was obtained from Bruno Stieger and Peter Meier (University Hospital, Zurich, Switzerland) (Madon et al., 1997). All other chemicals and unlabeled compounds were purchased from Sigma-Aldrich (St. Louis, MO).

Expression of Human MRP2 and Rat Mrp2 in Insect Cells. Sf9 cells were cultured and infected with the recombinant baculovirus stocks as described earlier (Bakos et al., 2000).

Membrane Preparation. Membrane vesicle preparations expressing the human or rat form of ABCC2 (MRP2/Mrp2-Sf9) were prepared by Solvo Biotechnology (Szeged, Hungary) from baculovirus-infected Sf9 cells essentially as described previously (Bodo et al., 2003). Membrane protein content was determined using the BCA method (Pierce Biotechnology, Rockford, IL). The presence of the human and rat MRP2/Mrp2 transporter was confirmed with Western blotting, using the MRP2-specific monoclonal antibody, M_2III-5 (Alexis Corporation, Lausen, Switzerland).

Vesicular Transport Assay. Inside-out membrane vesicles were incubated in the presence or absence of 4 mM ATP. For $E_2$17βG vesicular transport, the measurements were carried out in 7.5 mM $MgCl_2$, 40 mM MOPS-Tris, pH 7.0, and 70 mM KCl at 37°C for 2 min. The uptake was linear in this time frame. The transport was stopped by the addition of cold wash buffer (40 mM MOPS-Tris, pH 7.0, and 70 mM KCl), and the samples were immediately transferred to class B glass fiber filters, 1-μM pore size (Millipore Corporation, Billerica, MA). Filters were washed with 200 μl of ice-cold wash buffer five times, and radioactivity retained on the filter was measured by liquid scintillation counting. ATP-dependent transport was calculated by subtracting the values obtained in the absence of ATP from those in the presence of ATP.

Preparation and Culture of Primary Human and Rat Hepatocytes. Human liver tissues were obtained from kidney transplant donors by a quali-

TABLE 1

Concentration of modulators causing maximal potentiation of $E_2$17βG transport in the assays used

	Modular Concentration/Maximal Effect			
	MRP2 VT	Rat Mrp2 VT	MRP2 Sandwich	Rat Mrp2 Sandwich
	μM/% of control			
Indomethacin	100/750	270/510	10/220	100/325
Probenecid	330/265	1100/280	50/158	100/130
Benzbromarone	10/430	11/155	1/182	10/155
Sulfasalazine	35/430	110/360	10/250	10/170

VT, vesicular transport.

fied medical staff member from the Transplantation and Surgical Clinic, Semmelweis University of Budapest, as rejected donor livers. The human livers were inappropriate for transplantation for any reason. Permission of the Hungarian Regional Committee of Science and Research Ethics was obtained to use human tissues for scientific purposes. All studies involving human tissue followed the tenets of the Declaration of Helsinki. Hepatocytes were prepared by a three-step perfusion procedure. Human liver samples were first flushed with Ca^{2+}-free Earle's balanced salt solution containing EGTA and then with the same buffer without chelating agent and finally with Earle's balanced salt solution containing Ca^{2+} and type IV collagenase (Sigma-Aldrich). Perfusions were carried out at 37°C, pH 7.4, as described by Bayliss and Skett (1996). Rat hepatocytes were prepared from male Wistar rats (200–250 g) (Charles River, Budapest, Hungary) similarly to the method described above. Cell viability (>90%) was determined by trypan blue exclusion. All procedures were approved by the institutional animal care and use committee.

Hepatocytes were plated at a density of 2×10^6 cells/dish in 30-mm Petri dishes precoated with 0.15 ml of rat tail collagen type I solution (1.6 mg/ml) in Williams' Medium E containing 5% of fetal calf serum, 100 nM insulin, 2.5 μg/ml amphotericin B, 0.1 mg/ml gentamicin, 30 nM Na_2SeO_3, and 0.1 μM dexamethasone. The medium was aspirated, and cells were overlaid with 200 μl of ice-cold, neutralized rat tail collagen type I solution (1.5 mg/ml, pH 7.4) 24 h after plating, to achieve sandwich configuration. Williams' Medium E supplemented with insulin, gentamicin, dexamethasone, and Na_2SeO_3 was placed on the top of the gelled collagen layer 45 min after overlay.

MRP2/Mrp2 Transport Assay. Efflux studies in sandwich-cultured hepatocytes were performed by the modified method of Liu et al. (1999). In brief, hepatocytes cultured in a sandwich configuration for 48 h (rat) or 5 days (human) were incubated with 0.5 ml of 1 μM [^{3}H]$E_2$17βG for 10 min at 37°C in humidified atmosphere of 95% air-5% CO_2. Then the loading medium was removed, and the cells were rinsed three times with 2.0 ml of ice-cold standard or Ca^{2+},Mg^{2+}-free HBSS and incubated with 0.5 ml of standard or Ca^{2+},Mg^{2+}-free HBSS supplemented with the modulator compounds or the vehicle for 20 min. The modulators were present only in the efflux period of the experiments to avoid alteration of substrate uptake. The amount of $E_2$17βG in the efflux medium was analyzed by scintillation counting. The transport of $E_2$17βG into the canalicular networks was determined by subtracting the amount of $E_2$17βG in standard HBSS from that in the Ca^{2+},Mg^{2+}-free HBSS. The nonspecific [^{3}H]$E_2$17βG binding was taken into consideration by subtracting radioactivity measured in the efflux medium of Petri dishes with two collagen layers and without hepatocytes from that obtained in the presence of hepatocytes.

In Vivo Studies. Male Wistar rats (Charles River) weighing 250 to 300 g were used for in vivo Mrp2 interaction studies. All procedures were approved by the institutional animal care and use committee. The rats had free access to general food and water and were maintained in a temperature-controlled facility with a 12-h light/dark cycle for at least 1 week. Before the experiment was started, the animals were fasted overnight but were allowed free access to water. Under urethane anesthesia (1 g/kg i.p.) the common bile duct was cannulated with a polyethylene (PE-10) tube after laparotomy. Saline solution (2 ml s.c.) was administered every hour to maintain liquid equilibrium of rats. After the experiments, the rats were sacrificed by cardiac puncture under anesthesia.

Treatment of rats was started after 30 min of surgery. Tracer doses of [^{3}H]$E_2$17βG in 300 μl of saline were coadministered intraperitoneally with the modulator compounds. Control rats received the [^{3}H]$E_2$17βG and the vehicle only. Bile samples were collected every 10 min for 120 min, every 20 min 2 more hours, and every 30 min for an addition hour into preweighed tubes. Then the amount of $E_2$17βG in the bile samples was determined by scintillation counting.

Data Analysis. Vesicular transport assays were run in duplicates. Data are presented as mean ± S.D. For data analysis, GraphPad Prism (version 4.0; GraphPad Software Inc., San Diego, CA) was applied according to the equations as described in the following. K_m and V_{max} values from direct transport measurements were calculated using the Michaelis-Menten equation, after estimating the number of binding sites from the Hill plot:

$$V = \frac{V_{max}\, x[S]}{[S] + K_m}$$

where V is velocity (picomoles of substrate per milligram of protein per minute), V_{max} is maximal velocity, $[S]$ is substrate concentration (micromolar), and K_m is the Michaelis-Menten constant. The results of the competition-type vesicular transport assays were analyzed using the Hill equation (variable slope sigmoid equation):

$$V = V_{min} + \frac{V_{max} - V_{min}}{1 + 10^{(\log EC_{50} + [A])\, x n_H}}$$

where V is velocity (picomoles of substrate per milligram of protein per minute), V_{min} is minimal velocity (fully inhibited transport), V_{max} is maximal velocity (in the absence of inhibitor), EC_{50} is the ligand concentration producing 50% of maximal response (efficacy), $[A]$ is the actual test drug concentration, and the Hill slope is the parameter characterizing the degree of cooperativity.

Results

Kinetics of Human and Rat MRP2/Mrp2-Mediated $E_2$17βG Transport in Vesicular Transport Assay. Figure 1 shows the concentration dependence of human and rat MRP2/Mrp2-mediated $E_2$17βG transport. The transport follows classic Michaelis-Menten kinetics for rat Mrp2 (Fig. 1C), whereas a rather sigmoid shaped curve characterizes the transport by the human form (Fig. 1A), an indication for the presence of cooperative binding sites. The Hill numbers calculated are 1.58 and 0.98 for the human and the rat protein, respectively (Fig. 1, B and D). The $K_{0.5}$ value determined for MRP2 is ~150 μM, whereas in the presence of 100 μM indomethacin the saturation curve of human MRP2 becomes a Michaelis-Menten-type hyperbolic curve, with a K_m value of 139 μM and a Hill number of 1.04. At the same time the K_m value of the rat Mrp2 curve shifts from 61.5 to 26.3 μM in the presence of 100 μM indomethacin, whereas no change was observed in the Hill number. The experiment was repeated in the presence of 50 μM indomethacin at lower $E_2$17βG concentrations and for rat Mrp2, and the tendency of decreasing K_m value with increasing indomethacin concentration was confirmed (Fig. 1C, inset; K_m = 32.6 μM).

Table 1 and Fig. 2 summarize the results of the human and rat MRP2/Mrp2-mediated $E_2$17βG transport in the presence of different modulator molecules. These compounds dose dependently potentiated

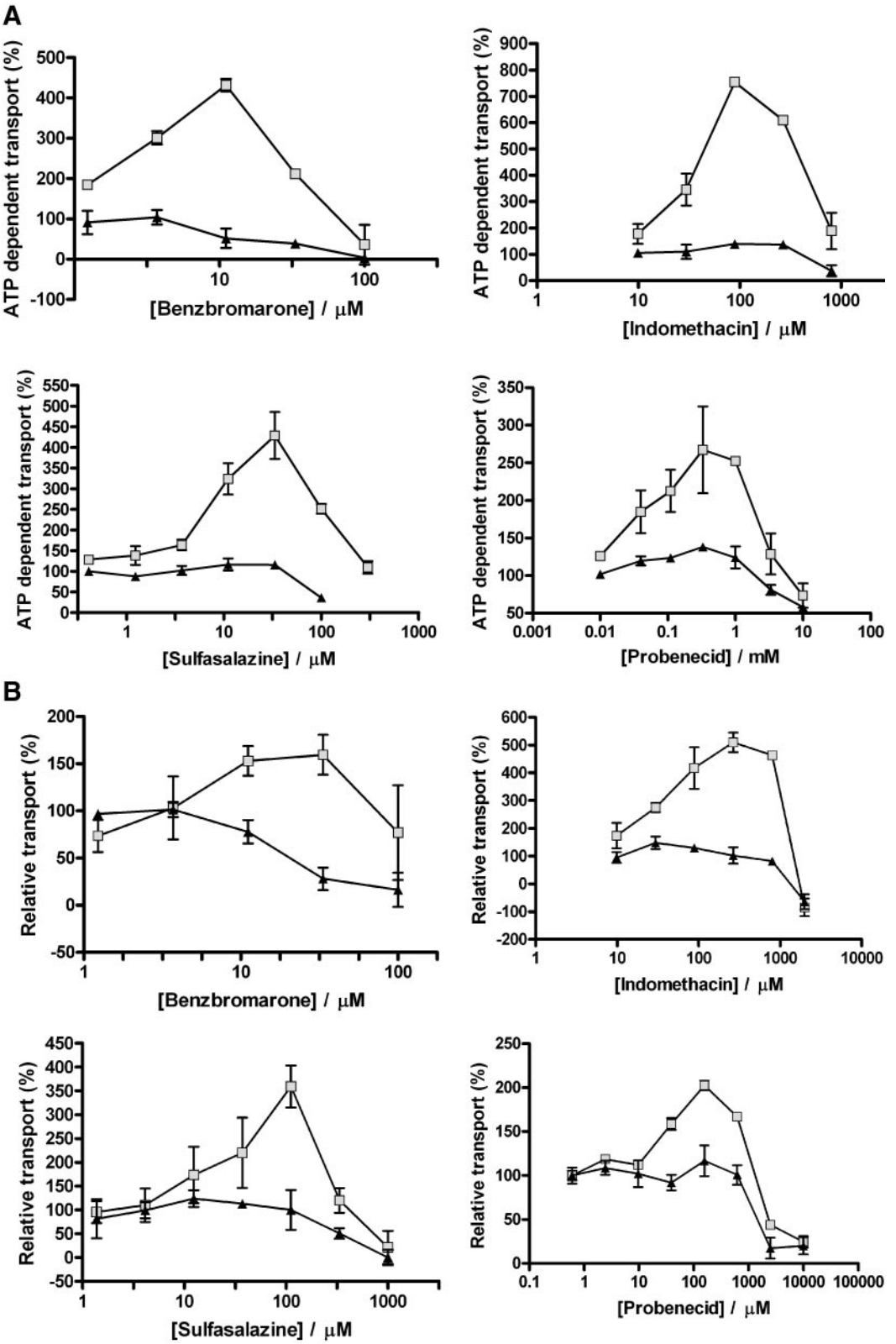

FIG. 2. Vesicular transport of $E_2$17βG by human and rat MRP2/Mrp2 transporter in the presence of modulators. A, effect of modulators on the transport of E217βG by MRP2. The effect is investigated in the presence of 1 μM E217βG (▨) and 50 μM E217βG (▲). B, effect of modulators on the transport of E217βG by rat Mrp2. The effect is investigated in the presence of 1 μM E217βG (▨) and 50 μM E217βG (▲).

the transport of $E_2$17βG. In both species, indomethacin proved to be the most efficacious modulator of $E_2$17βG transport among the compounds investigated, followed by sulfasalazine and probenecid. In these cases no large differences were observed between the maximal stimulatory effects relative to control values. Benzbromarone also stimulated the transport of $E_2$17βG in both species; however, the potentiation was more pronounced for the human protein compared with the rat protein (430% versus 155%).

Effect of Modulator Drugs on $E_2$17βG Transport Measured in Sandwich-Cultured Human and Rat Hepatocytes. Figure 3 shows the canalicular efflux of $E_2$17βG in sandwich-cultured rat and human hepatocytes in the presence of different concentrations of modulators.

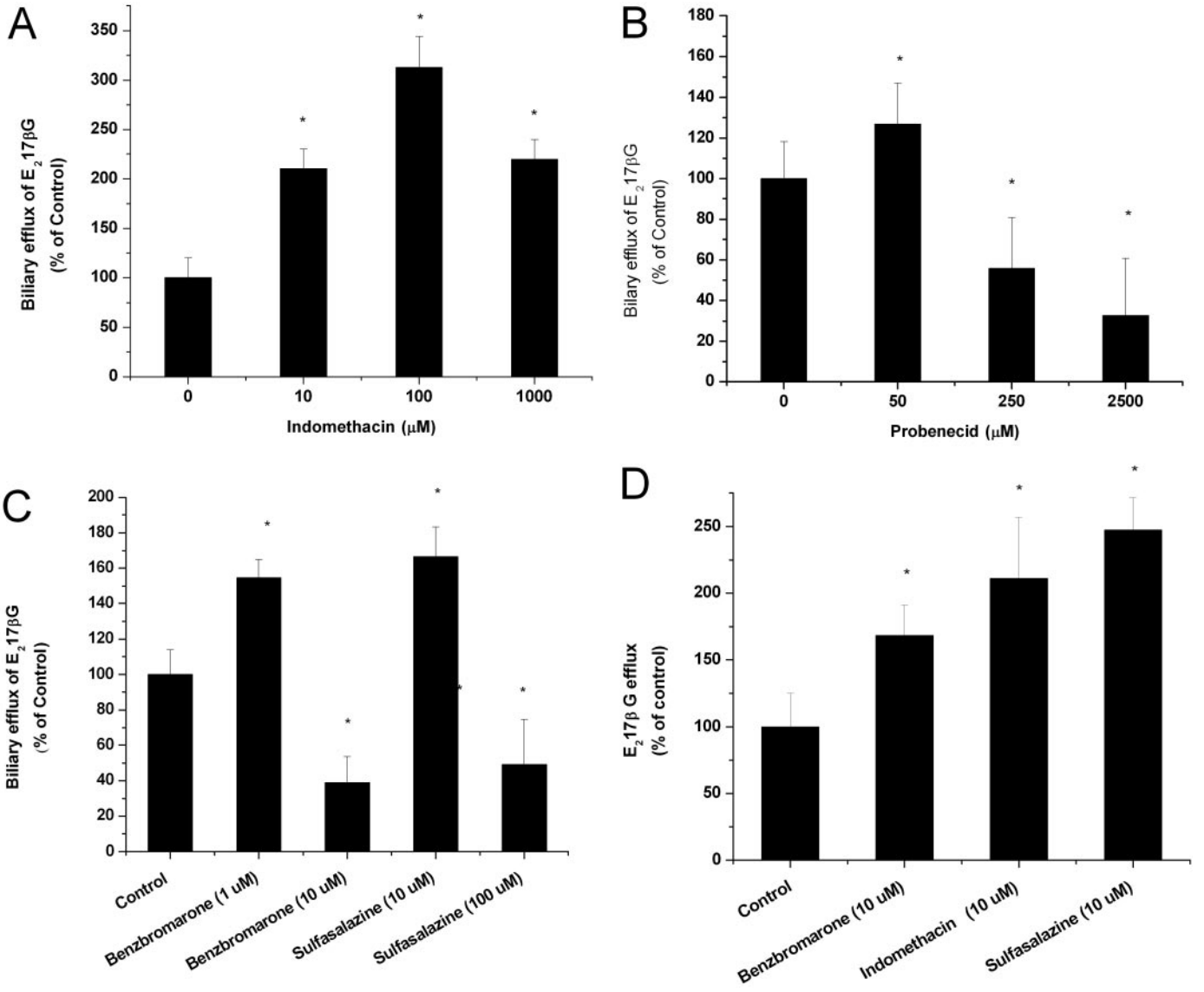

FIG. 3. Transporter mediated canalicular efflux of $E_2$17βG in the presence of modulators. Effect of modulators on the Mrp2-mediated canalicular efflux of $E_2$17βG in sandwich-cultured rat (A–C) or human (D) hepatocytes. Experiments were performed at 1 μM $E_2$17βG concentration. Data are expressed as a percentage of control, means ± S.D. ($n = 3$). All experiments were performed with hepatocytes from three independent preparations. *, significant difference compared with control ($p < 0.05$, determined by Student's t test).

Because the substrate uptake occurred in the absence of the modulators, these compounds could modulate the efflux transporters only. In rat hepatocytes indomethacin significantly elevated the canalicular transport of $E_2$17βG at all concentrations (Fig. 2A). Probenecid (Fig. 2B) and benzbromarone and sulfasalazine (Fig. 2C) potentiated the transport at low concentrations (50, 1, and 10 μM, respectively), but at higher concentrations (250 and 2500 μM and 10 and 100 μM, respectively) inhibited the biliary transport of $E_2$17βG. Likewise, in human hepatocyte cultures benzbromarone (10 μM), indomethacin (10 μM), and sulfasalazine (10 μM) potentiated the biliary efflux of $E_2$17βG (Fig. 2D).

In Vivo Efflux Experiments. Indomethacin significantly increased the efflux of $E_2$17βG without influencing the bile flow at a 5 mg/kg dose. The half-life was decreased by 40% (Fig. 4A). Indomethacin significantly increased the biliary efflux of $E_2$17βG shortly after administration, as shown in Fig. 4B.

Benzbromarone also increased the biliary elimination of $E_2$17βG (Fig. 4C). The half-life of $E_2$17βG decreased significantly even at a dose of 10 mg/kg, which could be further decreased by higher doses. Similar to the observations with indomethacin, the elimination rate peaked shortly after administration of benzbromarone (Fig. 4D).

Probenecid significantly decreased the half-life of $E_2$17βG at a 25 mg/kg dose compared with control, but at a higher dose (50 mg/kg) the difference was not significant (Fig. 4E). Probenecid is choleretic even at a 25 mg/kg dose (Fig. 4F), which may explain the disappearance of the effect. Alternatively, the effect of probenecid is bell-shaped in vivo, just as it is in vitro in the vesicular transport assay (Fig. 2B).

DISCUSSION

MRP2 transports many anionic drugs and drug metabolites that may interfere with the transport of endogenous MRP2 substrates, such as bilirubin glucuronide or $E_2$17βG (Bode et al., 2002). In this article known MRP2 interactors were investigated for their effect on the transport of $E_2$17βG. We selected three different assay systems, vesicular transport assays, hepatocyte sandwich culture experiments

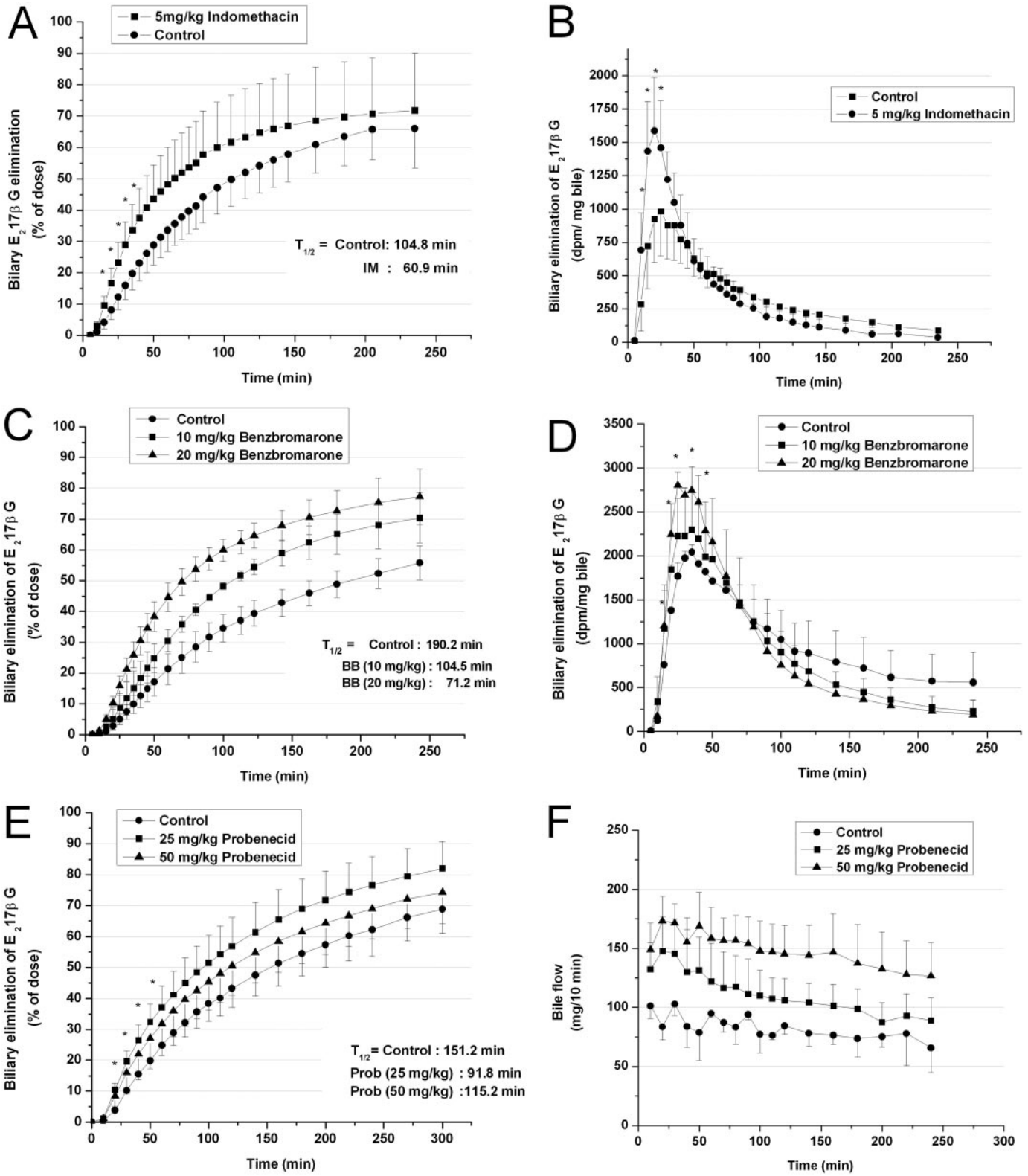

FIG. 4. Effect of modulators indomethacin (A and B), benzbromarone (C and D), and probenecid (E and F) on the biliary clearance of $E_2$17βG studied in vivo using rats. Each point represents the mean value ± S.D. (n = 3). *, significant difference compared with control ($p < 0.05$, determined by Student's t test).

to study differences in species specificity between human and rat, and in vivo rat studies to determine in vitro-in vivo correlations for rats.

Vesicular transport studies have shown a marked difference in the kinetics of the transport of $E_2$17βG, an important endogenous substrate of MRP2/Mrp2. The sigmoid transport curve and Hill plot data have shown that the human protein probably has two cooperative binding sites (n = 1.58), whereas the rat protein displayed noncooperative transport with a Hill number close to 1 (n = 0.98). There is a great deal of disagreement in data published on the MRP2/Mrp2-mediated $E_2$17βG transport (reviewed in Borst et al., 2006b). Single

digit K_m values were reported by a study (Cui et al., 1999) with Michaelis-Menten type kinetics for the human protein. The study by Zelcer et al. (2003), similarly to our data, showed cooperative interaction with lower affinities ($K_{0.5}$ = 120 μM). A study using human canalicular membrane vesicles showed Michaelis-Menten type kinetics with a K_m of 364 μM (Shilling et al., 2006). For rat protein classic Michaelis-Menten type kinetics was shown (Borst et al., 2006b). In contrast, two laboratories reported cooperative transport of $E_2$17βG by rat Mrp2. One of them demonstrated a sigmoidal transport with a Hill number of 1.16 (Ninomiya et al., 2005; also reviewed in Borst et al., 2006b), whereas the other group calculated a Hill number of 1.5 (Gerk et al., 2004). The reported K_m values range from single digit (Cui et al., 1999; Ito et al., 2001) through double digit (Borst et al., 2006b) to triple digit numbers (Shilling et al., 2006). Our data are similar to results obtained using rat Mrp2-Sf9 for which Michaelis-Menten type kinetics and double digit K_m (K_m = 16 μM) was found (Borst et al., 2006b).

We have shown that the phenomenon of cooperativity is not restricted to the human protein because a heterotropic effect on rat Mrp2 $E_2$17βG transport by many compounds is clearly seen (Fig. 2). The effect of modulators is more dramatic on the human MRP2-mediated $E_2$17βG transport as shown in Fig. 1A in which indomethacin converts the sigmoidal transport kinetics into hyperbolic kinetics. The rat Mrp2-mediated transport follows Michaelis-Menten kinetics rather than a sigmoid type (Fig. 1C). However, in the presence of 100 μM indomethacin, the K_m value decreases from 61.5 to 26.3 μM, explaining the potentiation phenomenon observed in the vesicular transport assay (Fig. 2).

It has been suggested (Borst et al., 2006b) that the differences observed in the rat Mrp2 data are due to differences in the membrane lipid composition. However, we have repeated $E_2$17βG transport using MDCKII membranes overexpressing rat Mrp2 and obtained Michaelis-Menten type kinetics (data not shown). Likewise, one of the articles cited (Shilling et al., 2006) used rat canalicular membrane vesicles membranes and observed hyperbolic kinetics.

In general, we have seen that the maximal stimulatory concentrations are higher for the rat transporter than those for the human transporter. It is also evident that the compounds have lower affinity for the modulating site on the rat protein than for the equivalent site on human MRP2 (Table 1). Differences in cooperativity have been found for MRP2 transporters from rat and dog (Ninomiya et al., 2005) and human and mouse (Zimmermann et al., 2008). The potentiating effect of different substrates depends on the concentration of $E_2$17βG. At 1 μM $E_2$17βG potentiation is seen, whereas at higher drug concentrations inhibition is seen (Fig. 4). At 50 μM $E_2$17βG only inhibition is seen (Fig. 2). With the two-site model proposed earlier (Zelcer et al., 2003), it can be envisioned that at lower (1 μM) $E_2$17βG concentrations the site used for the heterotropic effect is available for the modulator drugs. At greater (50 μM) $E_2$17βG concentrations both sites are occupied by the substrate $E_2$17βG. At greater drug concentrations, however, inhibition takes place without the potentiation phase of lower drug concentration, which suggests that at these respective substrate and modulator concentrations the modulator competes efficiently with $E_2$17βG for the transport site. These observations should be taken into consideration in the design of drug-transporter interaction assays.

The phenomenon of cooperativity in MRP2/Mrp2-mediated transport is not unique to the vesicular system. The phenomenon has been demonstrated with transfected MDCKII cells (Zimmermann et al., 2008). Our data using various assay systems to investigate the transport of $E_2$17βG, the most thoroughly studied substrate, also substantiate the observation. The maximal potentiation values and concentrations of $E_2$17βG transport are summarized in Table 1. The concentrations used in the sandwich-cultured hepatocyte experiments are clinically relevant for all modulators, for indomethacin (Takeda et al., 2002), for probenecid (Dayton et al., 1963), for sulfasalazine (Yamasaki et al., 2008), and also for benzbromarone (Ito et al., 2004; product information for Urinorm, 2005; Torii Pharmaceutical Co. Ltd, Tokyo, Japan). The concentrations used in the vesicular transport studies are in the range used by other studies (Bodo et al., 2003; Zelcer et al., 2003). These concentrations model intracellular values. The hepatocyte uptakes of probenecid (Terasaki et al., 1986) and indomethacin (Morita et al., 2005) are at least partly transporter-mediated; thus, free intracellular concentrations may exceed the extracellular concentration of free dugs. Sulfasalazine is a compound with an extremely low passive permeability and its cellular uptake is inhibited by organic anion transport inhibitors (Liang et al., 2000). Therefore, it is likely that intracellular concentrations reach values shown to potentiate $E_2$17βG transport in the vesicular assay. The $E_2$17βG concentration of 1 μM used in the sandwich culture experiments is justified by the low micromolar K_m of $E_2$17βG uptake into hepatocytes and is within the range used in other studies (Brouwer et al., 1987; Shitara et al., 2003). Using another approach, Sasaki et al. (2004) correlated the in vivo biliary clearance and in vitro transcellular transport of $E_2$17βG, using rat organic anion-transporting peptide 4 (Slc21a10) and Mrp2 double-transfected MDCKII cells, a hepatocyte model for vectorial transport studies. They found that extrapolation from in vitro data resulted in the underestimation of the in vivo blood to bile disposition. However, as the rate-determining step in both in vitro and in vivo systems was shown to be the uptake process, the role of Mrp2 has not been taken into consideration. Our experimental design differed 2-fold from this study, because we 1) used modulators that potentiated MRP2/Mrp2 activity and 2) separated the uptake process from the efflux step; hence, we were able to examine the efflux step. Our results indicate that the MRP2/Mrp2-mediated potentiation of $E_2$17βG transport shown in the vesicular transport assay is present in the hepatocyte sandwich culture experiments and in vivo for the rat transporter for all compounds tested.

In summary, we have demonstrated for the first time that $E_2$17βG transport potentiation by modulator drugs can also be observed in sandwich-cultured hepatocytes and in vivo biliary excretion experiments. Our data clearly show that the phenomenon of heterotropic cooperativity is physiologically and pharmacologically relevant. However, further studies are needed to establish the physiological and pharmacological significance of the phenomenon in vivo.

Acknowledgments. We are grateful to Drs. Balazs Sarkadi, András Váradi, and Bruno Stieger for providing the baculoviruses used in this work. The expert help of Judit Janossy, MSc, in reviewing and preparation of the manuscript is acknowledged.

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