

**1. Title page:**

**MRP2-mediated E<sub>2</sub>17βG transport potentiation: *in vitro* – *in vivo* correlation and species specificity\***

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**2. Running title page: Potentiation of MRP2-mediated E<sub>2</sub>17 $\beta$ G transport**

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List of non-standard abbreviations:

E<sub>2</sub>17 $\beta$ G : Estradiol-17- $\beta$ -D-glucuronide; MRP2 (ABCC2, cMOAT) multidrug-resistance protein 2; VT: vesicular transport; wt: wild type;

## ABSTRACT

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-17- $\beta$ -D-glucuronide (E<sub>2</sub>17 $\beta$ G), 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 non-substrates) potentiate the transport of E<sub>2</sub>17 $\beta$ G 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<sub>2</sub>17 $\beta$ G according to Michaelis-Menten type kinetics. Nevertheless, in the presence of modulator drugs E<sub>2</sub>17 $\beta$ G transport mediated by the rat transporter also shows cooperative kinetics as potentiation of E<sub>2</sub>17 $\beta$ G 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.

### 3. INTRODUCTION

MRP2 (ABCC2, cMOAT) is a member of the ABC 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, 2007). MRP2 has wide substrate specificity. Although it transports hydrophobic compounds in the presence of glutathione (Evers, 2000) its role in transporting anionic compounds and sulfate, glucuronide and glutathione conjugates is considered more important (König, 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 (Zelcer, 2006; Bode, 2002). Indeed, naturally occurring mutations leading to deficiencies of human MRP2 (Dubin-Johnson syndrome) and rat Mrp2 (TR-, Eisai hyperbilirubinemic rats) function (Wada et al. 1998; Toh, 1999; Paulusma, 1997; Kartenbeck, 1996; Buchler, 1996, respectively) 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 Elferink, 1997).

The transport of E<sub>2</sub>17βG, an MRP2 substrate (Keppler, 1997) does not follow the classical Michaelis-Menten kinetics (Bodó, 2003; Zelcer, 2003; Zimmermann, 2008).

The concentration dependence of transport follows rather sigmoid characteristics that can be explained by E<sub>2</sub>17βG binding to two different sites on MRP2. Zelcer *et al* referred to these sites as S (substrate) and M (modulator) and created four groups of MRP2

interactors based on their effect on E<sub>2</sub>17βG transport. There are modulator compounds that are able to stimulate E<sub>2</sub>17βG transport in a dose-dependent manner by competing with E<sub>2</sub>17βG for the M site, which manifests as an increased transport of this substrate. E<sub>2</sub>17βG is also a substrate of rat Mrp2 and this transporter mediates its biliary excretion (Morikawa, 2000). The transport kinetics of E<sub>2</sub>17βG by rat Mrp2 is controversial as both hyperbolic and sigmoid transport profiles have been reported (reviewed in Borst 2006a). Although most of these studies have been carried out in vesicular systems (reviewed in Borst, 2006a) the phenomenon has also been documented in cellular systems albeit using substrates other than E<sub>2</sub>17βG (Huisman, 2005; Zimmermann, 2008). However, the physiological relevance of this phenomenon is unclear, as the modulator-induced potentiation of Mrp2-mediated E<sub>2</sub>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 (Shilling, 2005; Ninomiya, 2005, Ninomiya, 2006; Zimmermann, 2008). However, detailed studies that included membrane as well as cellular experimental systems have only been performed for the human and the mouse protein (Zimmermann, 2008).

The present study investigates 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 is application of the vesicular

system and the sandwich cultured hepatocytes to reveal differences between transporter orthologs of human and rat origin.

#### 4. MATERIALS AND METHODS

**Materials.**  $^3\text{H}$ -Estradiol-17- $\beta$ -D glucuronide ( $\text{E}_217\beta\text{G}$ ) was purchased from Perkin Elmer (Boston, MA, USA). Recombinant baculovirus encoding wild-type human MRP2 was kind gifts 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 (U. Hospital, Zurich, Switzerland) (Madon, 1997). All other chemicals and unlabeled compounds were purchased from Sigma.

**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, 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 (Bodó, 2000). 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 blot, using the MRP2 specific monoclonal antibody, M<sub>2</sub>III-5 (Alexis Corp., Lausen, Switzerland).

**Vesicular Transport Assay.** Inside-out membrane vesicles were incubated in the presence or absence of 4 mM ATP. For  $\text{E}_217\beta\text{G}$  vesicular transport, the measurements were carried out in 7.5 mM  $\text{MgCl}_2$ , 40 mM MOPS-Tris, pH 7.0 and 70 mM KCl at 37°C

for 2 minutes. The uptake was linear in this timeframe. The transport was stopped by the addition of cold wash buffer (40 mM MOPS-Tris, pH 7.0, 70 mM KCl) and the samples were immediately transferred to class B glass fiber filters, 1  $\mu$ M pore size (Millipore, Billerica, MA, USA). Filters were washed with 200  $\mu$ l of ice-cold wash buffer 5-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 qualified medical staff from Transplantation and Surgical Clinic, Semmelweis University of Budapest as rejected donor livers. 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  $\text{Ca}^{2+}$  free Earle's balanced salt solution (EBSS) containing EGTA, then with the same buffer without chelating agent and finally with EBSS containing  $\text{Ca}^{2+}$  and type IV collagenase (Sigma-Aldrich, St. Louis, MO). Perfusions were carried out at 37<sup>0</sup> C, pH 7.4 as described by Bayliss and Skett (1996). Rat hepatocytes were prepared from male Wistar rats (200-250g) (Charles River, Budapest) similarly as 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  $2 \times 10^6$  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<sub>2</sub>SeO<sub>3</sub>, 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, Na<sub>2</sub>SeO<sub>3</sub> 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). Briefly, hepatocytes cultured in a sandwich configuration for 48 h (rat) or 5 days (human) were incubated with 0.5 ml of 1 µM <sup>3</sup>H E<sub>2</sub>17βG for 10 min at 37 °C in humidified atmosphere of 95% air-5% CO<sub>2</sub>. Then the loading medium was removed, and the cells were rinsed three times with 2.0 ml of ice cold standard or Ca<sup>2+</sup>, Mg<sup>2+</sup>-free HBSS and incubated with 0.5 ml of standard or Ca<sup>2+</sup>, Mg<sup>2+</sup>-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 in order to avoid alteration of substrate uptake. The amount of E<sub>2</sub>17βG in the efflux medium was analyzed by scintillation counting. The transport of E<sub>2</sub>17βG into the canalicular networks was determined by subtracting the amount of E<sub>2</sub>17βG in standard HBSS from that in the Ca<sup>2+</sup>, Mg<sup>2+</sup>-free HBSS. The nonspecific <sup>3</sup>H-E<sub>2</sub>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, Budapest) 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 starting the experiment, 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 PE-10 tubing after laparotomy. 2 ml of saline solution was administered sc. in 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  $^3\text{H-E}_2\text{17}\beta\text{G}$  in 300  $\mu\text{l}$  of saline was coadministered with the modulator compounds i.p. Control rats received the  $^3\text{H-E}_2\text{17}\beta\text{G}$  and the vehicle only. Bile samples were collected every 10 min for 120 min, then every 20 min two more hours, and every 30 min an addition hour into preweighed tubes. Then the amount of  $\text{E}_2\text{17}\beta\text{G}$  in the bile samples was determined by scintillation counting.

**Data analysis.** Vesicular transport assays were run in duplicates. Data are presented as mean  $\pm$  S.D. For data analysis, the GraphPad PRISM 4.0 software (GraphPad Software Inc., San Diego, CA) was applied, using the following equations:

$K_m$  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 (pmol substrate per mg protein per minute),  $V_{\max}$  is maximal velocity,  $[S]$  is substrate concentration in  $\mu\text{M}$  and  $K_m$  is Michaelis-Menten constant.

The results of the competition type vesicular transport assays were analyzed using the Hill equation (variable slope sigmoid equation):

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

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

## 5. RESULTS

### **Kinetics of human and rat MRP2/Mrp2-mediated E<sub>2</sub>17 $\beta$ G transport in vesicular transport assay**

Figure 1 shows the concentration dependence of human and rat MRP2/Mrp2 mediated E<sub>2</sub>17 $\beta$ G transport. The transport follows classical Michaelis-Menten kinetics in case of rat Mrp2 (Figure 1/C), while a rather sigmoid shaped curve characterizes the transport by the human form (Figure 1/A), 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  $\sim 150 \mu\text{M}$ , while in the presence of  $100 \mu\text{M}$  indomethacin the saturation curve of human MRP2 becomes a Michaelis-Menten-type hyperbolic one, with a  $K_m$  value of  $139 \mu\text{M}$  and a Hill number of

1.04. At the same time the  $K_m$  value of the rat Mrp2 curve shifts from 61.5  $\mu\text{M}$  to 26.3  $\mu\text{M}$  in the presence of 100  $\mu\text{M}$  indomethacin, while no change was observed in the Hill number. The experiment was repeated in the presence of 50  $\mu\text{M}$  indomethacin at lower  $E_217\beta\text{G}$  concentrations as well in case of rat Mrp2 and the tendency of decreasing  $K_m$  value with increasing indomethacin concentration was confirmed (Figure 1/C – inset;  $K_m = 32.6 \mu\text{M}$ ).

Table 1 summarizes the results of the human and rat MRP2/Mrp2 mediated  $E_217\beta\text{G}$  transport in the presence of different modulator molecules. These compounds dose-dependently potentiated the transport of  $E_217\beta\text{G}$ . In both species, indomethacin proved to be the most efficacious modulator of  $E_217\beta\text{G}$  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  $E_217\beta\text{G}$  in both species, however, the potentiation was more pronounced in case of the human protein compared to the rat one (430% versus 155%).

### **Effect of Modulator drugs on $E_217\beta\text{G}$ transport measured in sandwich cultured human and rat hepatocytes**

Figure 3 shows the canalicular efflux of  $E_217\beta\text{G}$  in sandwich cultured rat and human hepatocytes in the presence of different concentrations of modulators. Since 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  $E_217\beta\text{G}$  at all concentrations (Fig.3/A). Probenecid (3/B) and

benzbromarone, sulfasalazine (3/C) potentiated the transport at low concentrations (50  $\mu\text{M}$ , 1  $\mu\text{M}$ , 10  $\mu\text{M}$ , respectively), however, at higher concentrations (250  $\mu\text{M}$  and 2500  $\mu\text{M}$ ; 10  $\mu\text{M}$ ; 100  $\mu\text{M}$ ; respectively) inhibited the biliary transport of  $\text{E}_217\beta\text{G}$ . Similarly, in human hepatocyte cultures benzbromarone (10  $\mu\text{M}$ ), indomethacin (10  $\mu\text{M}$ ) and sulfasalazine (10  $\mu\text{M}$ ) potentiated the biliary efflux of  $\text{E}_217\beta\text{G}$  (Fig.3/D).

### ***In vivo* efflux experiments**

Indomethacin significantly increased the efflux of  $\text{E}_217\beta\text{G}$  without influencing the bile flow at a 5 mg/kg dose. The half-life was decreased by 40% (Figure 4/A). Indomethacin significantly increased the biliary efflux of  $\text{E}_217\beta\text{G}$  shortly after administration, as shown on Figure 4/B.

Benzbromarone also increased the biliary elimination of  $\text{E}_217\beta\text{G}$  (Figure 4/C). The half-life of  $\text{E}_217\beta\text{G}$  decreased significantly even at a dose of 10 mg/kg, which could be further decreased by higher doses. Similarly to the observations with indomethacin the elimination rate peaked shortly after administration of benzbromarone (Figure 4/D).

Probenecid significantly decreased the half-life of  $\text{E}_217\beta\text{G}$  at a 25 mg/kg dose compared to control, but at a higher dose (50 mg/kg) the difference was not significant (Figure 4/E).

Probenecid is choleric even at a 25 mg/kg dose (Figure 4/F), 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 2/B).

## 6. 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<sub>2</sub>17βG (Bode, 2002). In this paper known MRP2 interactors were investigated for their effect on the transport of the latter substrate. We selected three different assay systems: the vesicular transport assay, the hepatocyte sandwich culture experiments to study differences in species specificity between human and rat, and performed *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<sub>2</sub>17βG, an important endogenous substrate of MRP2/Mrp2. The sigmoid transport curve and Hill plot data have shown that the human protein likely has two cooperative binding sites ( $n = 1.58$ ), while the rat protein displayed non-cooperative 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<sub>2</sub>17βG transport (reviewed in Borst, 2006b). Single digit  $K_m$  values were reported by a study (Cui, 1999) with Michaelis-Menten type kinetics for the human protein. The study by Zelcer *et al* (2003) similarly to our data has found a cooperative interaction with lower affinities ( $K_{0.5} = 120 \mu\text{M}$ ). The study utilizing human CMV showed Michaelis-Menten type kinetics with a  $K_m$  of 364  $\mu\text{M}$  (Shilling, 2006). For the rat protein a classical Michaelis-Menten type kinetics was shown (Borst, 2006b). In contrast, two labs reported cooperative transport of E<sub>2</sub>17βG by rat Mrp2. One of them demonstrated a sigmoidal transport with a Hill number of 1.16 (Ninomya, 2005; also reviewed in Borst, 2006b) while the other group calculated a Hill number of 1.5 (Gerk, 2004). The reported  $K_m$  values range from single digit (Cui, 1999;

Ito, 2001), through double digit (Borst, 2006b) to triple digit numbers (Shilling, 2006). Our data are similar to results obtained using rat Mrp2-Sf9 where Michaelis-Menten type kinetics and double digit  $K_m$  ( $K_m = 16 \mu\text{M}$ ) was found (Borst, 2006b).

We have shown that the phenomenon of cooperativity is not restricted to the human protein since a heterotropic effect on rat Mrp2  $E_217\beta\text{G}$  transport by many compounds is clearly seen (Fig. 2). The effect of modulators is more dramatic on the human MRP2-mediated  $E_217\beta\text{G}$  transport as shown in Fig. 1/A where indomethacin converts the sigmoidal transport kinetics into a hyperbolic one. The rat Mrp2-mediated transport follows Michaelis-Menten kinetics rather than a sigmoid type (Figure 1/C). However, in the presence of  $100 \mu\text{M}$  indomethacin, the  $K_m$  value decreases from  $61.5 \mu\text{M}$  to  $26.3 \mu\text{M}$ , explaining the potentiation phenomenon observed in the vesicular transport assay (Fig. 2).

It has been suggested (Borst, 2006b) that the differences observed in the rat Mrp2 data are due to differences in the membrane lipid composition. However, we have repeated  $E_217\beta\text{G}$  transport using MDCKII membranes overexpressing rat Mrp2 and obtained Michaelis-Menten type kinetics (data not shown). Similarly, one of the papers cited (Schilling, 2006) used rat CMV membranes and received hyperbolic kinetics.

In general, we have seen that the maximal stimulatory concentrations are higher in case of the rat transporter than those of 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 the human MRP2 (Table 1). Differences in cooperativity have been found for MRP2 transporters from rat and dog (Ninomiya, 2005) and human and mouse (Zimmermann, 2008). The potentiating effect of different substrates depends on the

concentration of E<sub>2</sub>17βG. At 1 μM E<sub>2</sub>17βG potentiation, while at higher drug concentrations inhibition is seen (Fig. 2). At 50 μM concentration of E<sub>2</sub>17βG only inhibition is seen (Fig. 2). With the two-site model proposed earlier (Zelcer, 2003) it can be envisioned that at lower (1 μM) E<sub>2</sub>17βG concentrations the site used for the heterotropic effect is available for the modulator drugs at lower concentration. At greater (50 μM) E<sub>2</sub>17βG concentrations both sites are occupied by the substrate E<sub>2</sub>17βG. At greater drug concentrations, however, inhibition takes place without the potentiation phase at lower drug concentration, which suggests that at this respective substrate – modulator concentrations the modulator competes efficiently with E<sub>2</sub>17βG for the transport site. These observations should be taken into consideration when designing 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, 2008). Our data using various assay systems to investigate the transport of E<sub>2</sub>17βG, the most thoroughly studied substrate also substantiate the observation. The maximal potentiation values and concentrations of E<sub>2</sub>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, 2002), probenecid (Dayton, 1963), sulfasalazine (Yamasaki, 2008) and also for benzbromarone (Ito, 2004; and product information by the producer Torii pharmaceutical Co. Ltd). The concentrations used in the vesicular transport studies are in the range used by other studies (Zelcer, 2003; Bodo, 2003). These concentrations model intracellular values. The hepatocyte uptake of probenecid (Terasaki, 1986) and indomethacin (Morita, 2005)

is at least partly transporter mediated, thus, free intracellular concentrations may exceed the extracellular concentration of free drugs. Sulfasalazine is a compound with an extremely low passive permeability and its cellular uptake is inhibited by organic anion transport inhibitors (Liang, 2000). It is likely therefore that intracellular concentrations reach values shown to potentiate E<sub>2</sub>17βG transport in vesicular assay. The E<sub>2</sub>17βG concentration of 1 μM used in the sandwich culture experiments is justified by the low micromolar K<sub>m</sub> of E<sub>2</sub>17βG uptake into hepatocytes and is within the range used in other studies (Brouwer 1987; Shitara 2003). Using another approach, Sasaki et al. (Sasaki, 2004) correlated the *in vivo* biliary clearance and *in vitro* transcellular transport of E<sub>2</sub>17βG, using rat Oatp4 (Slc21a10) and Mrp2 double transfected MDCKII cells, a hepatocyte model for vectorial transport studies. They have found that extrapolation from *in vitro* data resulted in the underestimation of *in vivo* blood to bile disposition. However, as the rate-determining step in both *in vitro* and *in vivo* systems was determined to be the uptake process, the role of Mrp2 has not been taken into consideration. Our experimental design differed twofold from this study, as (i) we used modulators that potentiated MRP2/Mrp2 activity and (ii) we 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<sub>2</sub>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<sub>2</sub>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*.

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## 8. References

- Bakos E, Evers R, Sinko E, Varadi A, Borst P and Sarkadi B (2000) Interactions of the human multidrug resistance proteins MRP1 and MRP2 with organic anions. *Mol Pharmacol* 57:760-8.
- Bayliss MK and Skett P (1996) *Human Cell Culture Protocols*. pp 369-89, Humana Press, New Jersey.
- Bode KA, Donner MG, Leier I and Keppler D (2002) Inhibition of transport across the hepatocyte canalicular membrane by the antibiotic fusidate. *Biochem Pharmacol* 64:151-8.
- Bodo A, Bakos E, Szeri F, Varadi A and Sarkadi B (2003) Differential modulation of the human liver conjugate transporters MRP2 and MRP3 by bile acids and organic anions. *J Biol Chem* 278:23529-37.
- Borst P, Zelcer N, van de Wetering K and Poolman B (2006a) On the putative co-transport of drugs by multidrug resistance proteins. *FEBS Lett* 580:1085-93.
- Borst P, Zelcer N and van de Wetering K (2006b) MRP2 and 3 in health and disease. *Cancer Lett* 234:51-61.
- Brouwer KL, Durham S, Vore M (1987) Multiple carriers for uptake of [3H]estradiol-17 beta(beta-D-glucuronide) in isolated rat hepatocytes. *Mol Pharmacol*. 32:519-23.
- Buchler M, Konig J, Brom M, Kartenbeck J, Spring H, Horie T and Keppler D (1996) cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. *J Biol Chem* 271:15091-8.

Cui Y, Konig J, Buchholz JK, Spring H, Leier I and Keppler D (1999) Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. *Mol Pharmacol* 55:929-37.

Dayton PG, Yu TF, Chen W, Berger L, West LA, Gutman AB (1963) The physiological disposition of probenecid, including renal clearance, in man, studied by an improved method for its estimation in biological material. *J Pharmacol Exp Ther* 140:278-86.

Evers R, de Haas M, Sparidans R, Beijnen J, Wielinga PR, Lankelma J and Borst P (2000) Vinblastine and sulfinpyrazone export by the multidrug resistance protein MRP2 is associated with glutathione export. *Br J Cancer* 83:375-83.

Gerk PM, Li W and Vore M (2004) Estradiol 3-glucuronide is transported by the multidrug resistance-associated protein 2 but does not activate the allosteric site bound by estradiol 17-glucuronide. *Drug Metab Dispos* 32:1139-45.

Huisman MT, Chhatta AA, van Tellingen O, Beijnen JH and Schinkel AH (2005) MRP2 (ABCC2) transports taxanes and confers paclitaxel resistance and both processes are stimulated by probenecid. *Int J Cancer* 116:824-9.

Ito K, Suzuki H and Sugiyama Y (2001) Charged amino acids in the transmembrane domains are involved in the determination of the substrate specificity of rat Mrp2. *Mol Pharmacol* 59:1077-85.

Kartenbeck J, Leuschner U, Mayer R and Keppler D (1996) Absence of the canalicular isoform of the MRP gene-encoded conjugate export pump from the hepatocytes in Dubin-Johnson syndrome. *Hepatology* 23:1061-6.

Kepler D, Leier I, Jedlitschky G (1997) Transport of glutathione conjugates and glucuronides by the multidrug resistance proteins MRP1 and MRP2. *Biol Chem* 378:787-91.

Konig J, Nies AT, Cui Y, Leier I and Kepler D (1999) Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. *Biochim Biophys Acta* 1461:377-94.

Liang E, Proudfoot J, Yazdanian M (200) Mechanisms of transport and structure-permeability relationship of sulfasalazine and its analogs in Caco-2 cell monolayers. *Pharm Res* 17:1168-74.

Liu X, LeCluyse EL, Brouwer KR, Gan LS, Lemasters JJ, Stieger B, Meier PJ, Brouwer KL (1999) Biliary excretion in primary rat hepatocytes cultured in a collagen-sandwich configuration. *Am J Physiol* 277(1 Pt 1):G12-21.

Madon J, Eckhardt U, Gerloff T, Stieger B and Meier PJ (1997) Functional expression of the rat liver canalicular isoform of the multidrug resistance-associated protein. *FEBS Lett* 406:75-8.

McGinnity DF, Tucker J, Trigg S, Riley RJ (2005) Prediction of CYP2C9-mediated drug-drug interactions: a comparison using data from recombinant enzymes and human hepatocytes *Drug Metab Dispos* 33:1700-7.

Morikawa A, Goto Y, Suzuki H, Hirohashi T and Sugiyama Y (2000) Biliary excretion of 17beta-estradiol 17beta-D-glucuronide is predominantly mediated by cMOAT/MRP2. *Pharm Res* 17:546-52.

Morita N, Kusuhara H, Nozaki Y, Endou H and Sugiyama Y (2005) Functional involvement of rat organic anion transporter 2 (Slc22a7) in the hepatic uptake of the nonsteroidal anti-inflammatory drug ketoprofen. *Drug Metab Dispos* 33:1151-7.

Neuhoff S, Ungell AL, Zamora I and Artursson P (2005) pH-Dependent passive and active transport of acidic drugs across Caco-2 cell monolayers. *Eur J Pharm Sci* 25:211-20.

Nies AT, Keppler D. (2007) The apical conjugate efflux pump ABCC2 (MRP2). *Pflugers Arch* 453:643-59.

Ninomiya M, Ito K and Horie T (2005) Functional analysis of dog multidrug resistance-associated protein 2 (Mrp2) in comparison with rat Mrp2. *Drug Metab Dispos* 33:225-32.

Ninomiya M, Ito K, Hiramatsu R and Horie T (2006) Functional analysis of mouse and monkey multidrug resistance-associated protein 2 (Mrp2). *Drug Metab Dispos* 34:2056-63.

Paulusma CC, Kool M, Bosma PJ, Scheffer GL, ter Borg F, Scheper RJ, Tytgat GN, Borst P, Baas F and Oude Elferink RP (1997) A mutation in the human canalicular multispecific organic anion transporter gene causes the Dubin-Johnson syndrome. *Hepatology* 25:1539-42.

Paulusma CC and Oude Elferink RP (1997) The canalicular multispecific organic anion transporter and conjugated hyperbilirubinemia in rat and man. *J Mol Med* 75:420-8.

Sasaki M, Suzuki H, Aoki J, Ito K, Meier PJ and Sugiyama Y (2004) Prediction of in vivo biliary clearance from the in vitro transcellular transport of organic anions across

a double-transfected Madin-Darby canine kidney II monolayer expressing both rat organic anion transporting polypeptide 4 and multidrug resistance associated protein 2. *Mol Pharmacol* 66:450-9.

Shilling AD, Azam F, Kao J and Leung L (2006) Use of canalicular membrane vesicles (CMVs) from rats, dogs, monkeys and humans to assess drug transport across the canalicular membrane. *J Pharmacol Toxicol Methods* 53:186-97.

Shitara Y, Li AP, Kato Y, Lu C, Ito K, Itoh T, Sugiyama Y Function of uptake transporters for taurocholate and estradiol 17beta-D-glucuronide in cryopreserved human hepatocytes. *Drug Metab Pharmacokinet.* 18:33-41.

Takeda M, Khamdang S, Narikawa S, Kimura H, Hosoyamada M, Cha SH, Sekine T, Endou H (2002) Characterization of methotrexate transport and its drug interactions with human organic anion transporters. *J Pharmacol Exp Ther* 302:666-71.

Terasaki T, Tamai I, Takanosu K, Nakashima E, Tsuji A (1986) Kinetic evidence for a common transport route of benzylpenicillin and probenecid by freshly prepared hepatocytes in rats. Influence of sodium ion, organic anions, amino acids and peptides on benzylpenicillin uptake *J Pharmacobiodyn* 9:18-28.

Toh S, Wada M, Uchiumi T, Inokuchi A, Makino Y, Horie Y, Adachi Y, Sakisaka S and Kuwano M (1999) Genomic structure of the canalicular multispecific organic anion-transporter gene (MRP2/cMOAT) and mutations in the ATP-binding-cassette region in Dubin-Johnson syndrome. *Am J Hum Genet* 64:739-46.

Wada M, Toh S, Taniguchi K, Nakamura T, Uchiumi T, Kohno K, Yoshida I, Kimura A, Sakisaka S, Adachi Y and Kuwano M (1998) Mutations in the canalicular multispecific organic anion transporter (cMOAT) gene, a novel ABC transporter, in

patients with hyperbilirubinemia II/Dubin-Johnson syndrome. *Hum Mol Genet* 7:203-7.

Yamasaki Y, Ieiri I, Kusuhara H, Sasaki T, Kimura M, Tabuchi H, Ando Y, Irie S, Ware J, Nakai Y, Higuchi S, Sugiyama Y. (2008) Pharmacogenetic characterization of sulfasalazine disposition based on NAT2 and ABCG2 (BCRP) gene polymorphisms in humans. *Clin Pharmacol Ther* 84:95-103.

Zelcer N, Huisman MT, Reid G, Wielinga P, Breedveld P, Kuil A, Knipscheer P, Schellens JH, Schinkel AH and Borst P (2003) Evidence for two interacting ligand binding sites in human multidrug resistance protein 2 (ATP binding cassette C2). *J Biol Chem* 278:23538-44.

Zelcer N, van de Wetering K, de Waart R, Scheffer GL, Marschall HU, Wielinga PR, Kuil A, Kunne C, Smith A, van der Valk M, Wijnholds J, Elferink RO and Borst P (2006) Mice lacking Mrp3 (Abcc3) have normal bile salt transport, but altered hepatic transport of endogenous glucuronides. *J Hepatol* 44:768-75.

Zimmermann C, van de Wetering K, van de Steeg E, Wagenaar E, Vens C and Schinkel AH (2008) Species-dependent transport and modulation properties of human and mouse multidrug resistance protein 2 (MRP2/Mrp2, ABCC2/Abcc2). *Drug Metab Dispos* 36:631-40.



## 10. Footnotes

\*Financial support

<sup>1</sup>Equally contributed to the study

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## 11. Legends for figures.

**Figure 1. Effect of indomethacin on the transport of E<sub>2</sub>17βG by human MRP2 (A, B) and rat Mrp2 (C, D).** In case of human MRP2 in the absence of 100 μM indomethacin (closed squares) the estimated apparent half-maximal rate (K<sub>0.5</sub>) was 150 μM, while in the presence of indomethacin (closed triangles), the K<sub>m</sub> is 139 μM and the V<sub>max</sub> is 1413 pmol/mg protein/min. K<sub>m</sub> and V<sub>max</sub> values for rat Mrp2 in the absence of indomethacin (closed squares) are 61.5 μM and 74.4 pmol/mg protein/min, respectively, while in the presence (closed triangles) 26.3 μM and 75.3 pmol/mg protein/min, respectively. Inset in panel C shows the effect of 50 μM indomethacin on E<sub>2</sub>17βG transport by rat Mrp2 at lower substrate concentrations (K<sub>m</sub> = 32.6 μM). Representative Hill plots are shown for human and rat protein in panels B and D, respectively, for transport in the absence (solid line) or in the presence (dotted line) of 100 μM indomethacin.

**Figure 2. Vesicular transport of E<sub>2</sub>17βG by human and rat MRP2/Mrp2 transporter in the presence of modulators.** A) Effect of modulators on the transport of E<sub>2</sub>17βG by MRP2. The effect is investigated in the presence of 1 μM E<sub>2</sub>17βG (gray squares) and 50 μM E<sub>2</sub>17βG (black triangles). B) Effect of modulators on the transport of E<sub>2</sub>17βG by rat Mrp2. The effect is investigated in the presence of 1 μM E<sub>2</sub>17βG (gray squares) and 50 μM E<sub>2</sub>17βG (black triangles).

**Figure 3. Transporter mediated canalicular efflux of E<sub>2</sub>17βG in the presence of modulators** Effect of modulators on the Mrp2-mediated canalicular efflux of E<sub>2</sub>17βG in

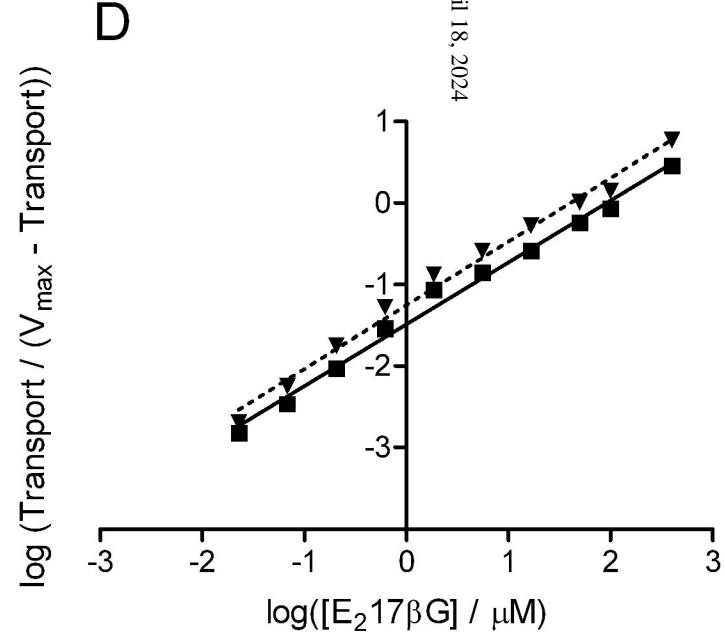
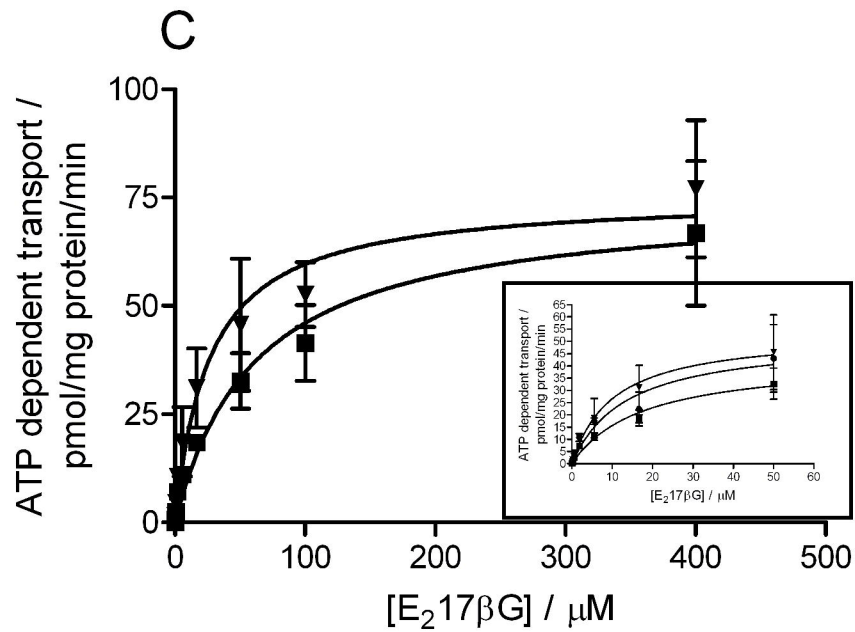
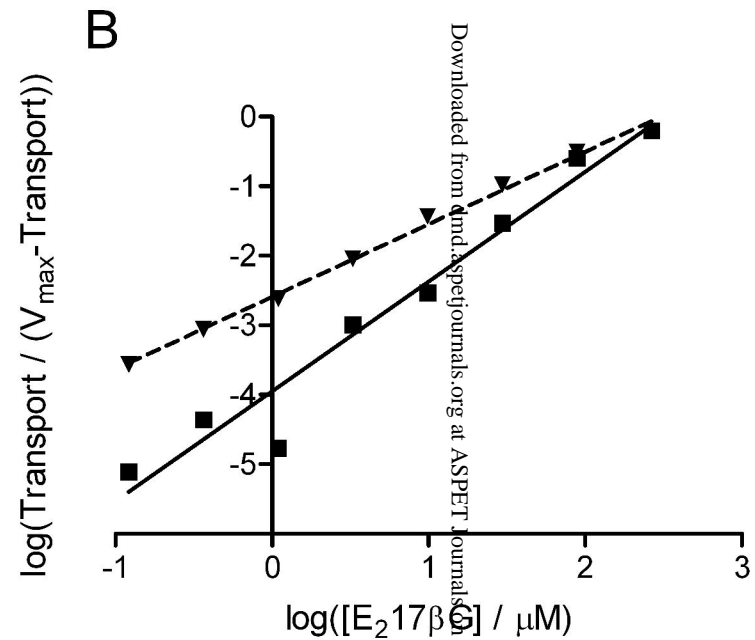
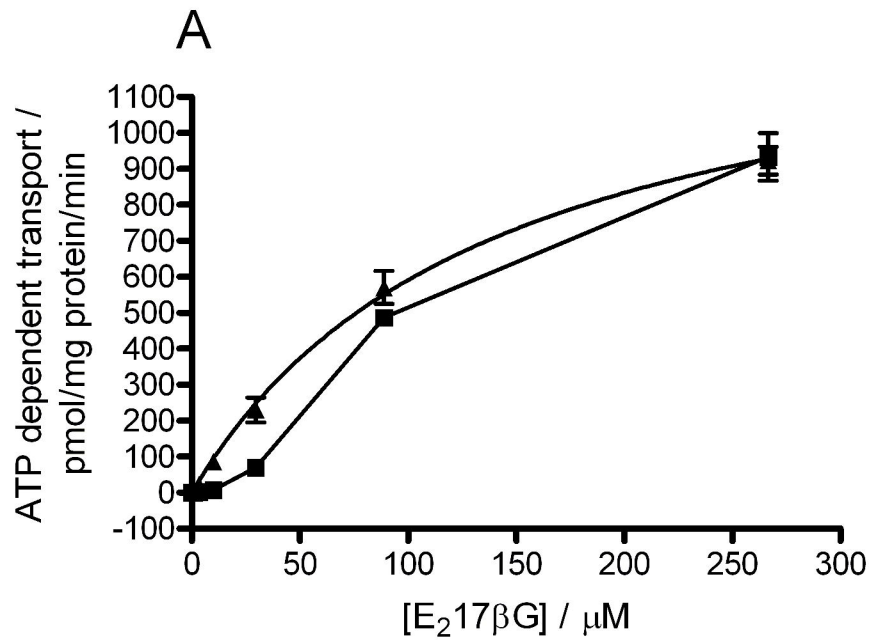
sandwich cultured rat (3A-C) or human (3D) hepatocytes. Experiments were performed at 1  $\mu$ M E<sub>2</sub>17 $\beta$ G concentration. Data are expressed as % of control, means  $\pm$  S.D. (n = 3). All experiments were performed with hepatocytes from 3 independent preparations. Asterisks (\*) indicate significant difference compared to control ( $p < 0.05$ , determined by Student's t test)

**Figure 4. Effect of modulators indomethacin (A,B), benzbromarone (C,D) and probenecid (E,F) on the biliary clearance of E<sub>2</sub>17 $\beta$ G studied *in vivo* using rats.**

Each point represents the mean value  $\pm$  S.D., for n = 3. Asterisks (\*) indicate significant difference compared to control ( $p < 0.05$ , determined by Student's test).

Table 1 Concentration of modulators ( $\mu\text{M}$ ) causing maximal potentiation (%) of  $\text{E}_217\beta\text{G}$  transport in the assays used

	MRP2 VT	rat Mrp2 VT	MRP2 sandwich	rat Mrp2 sandwich
	Modulator concentration ( $\mu\text{M}$ ) / maximal effect (% 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



**Figure 1.**

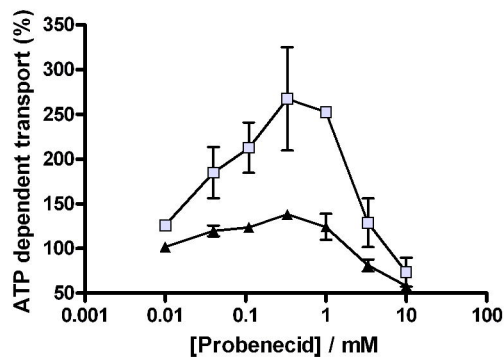
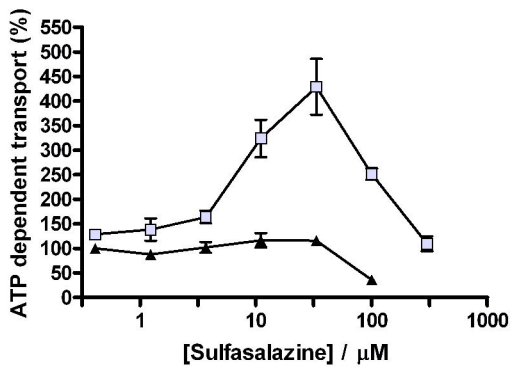
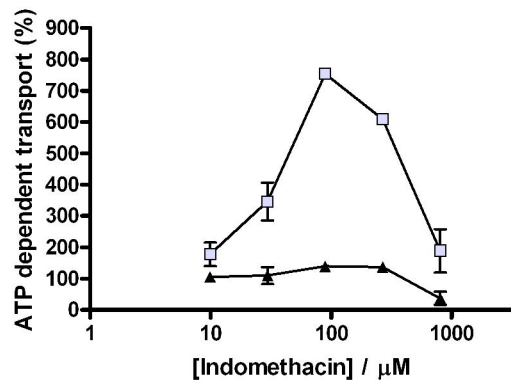
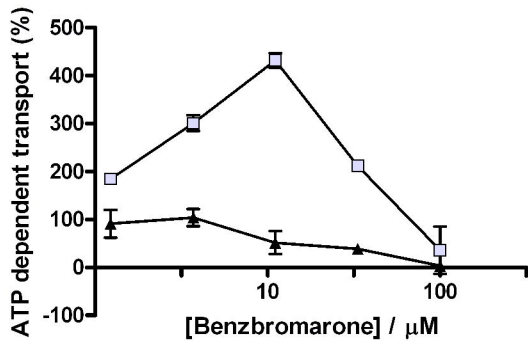


Figure 2/A

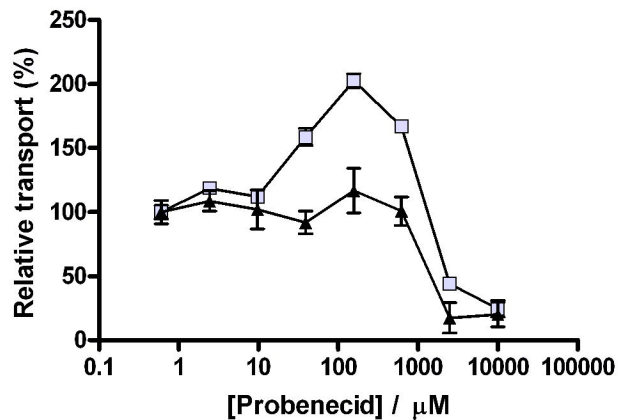
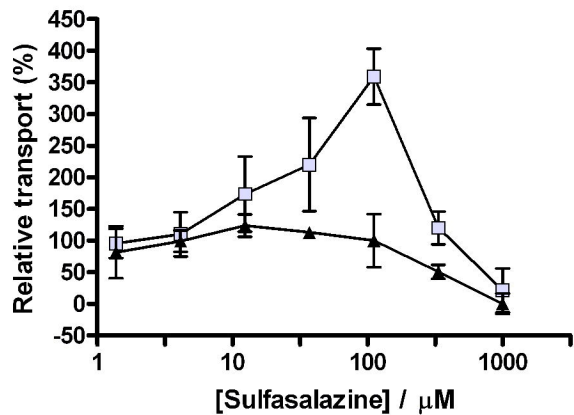
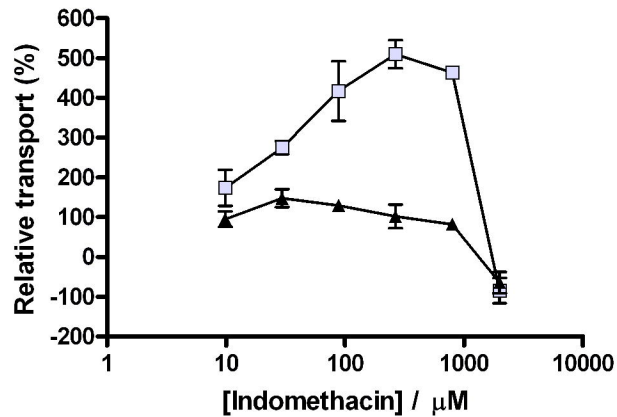
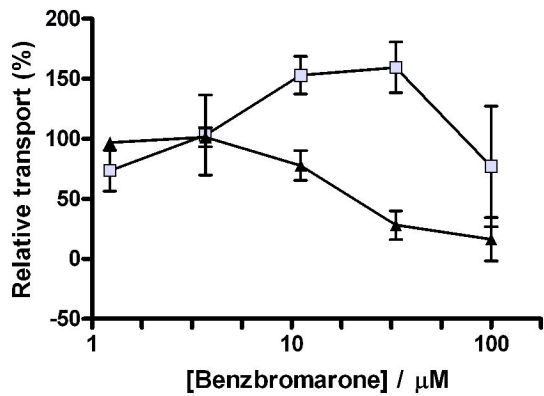


Figure 2/B

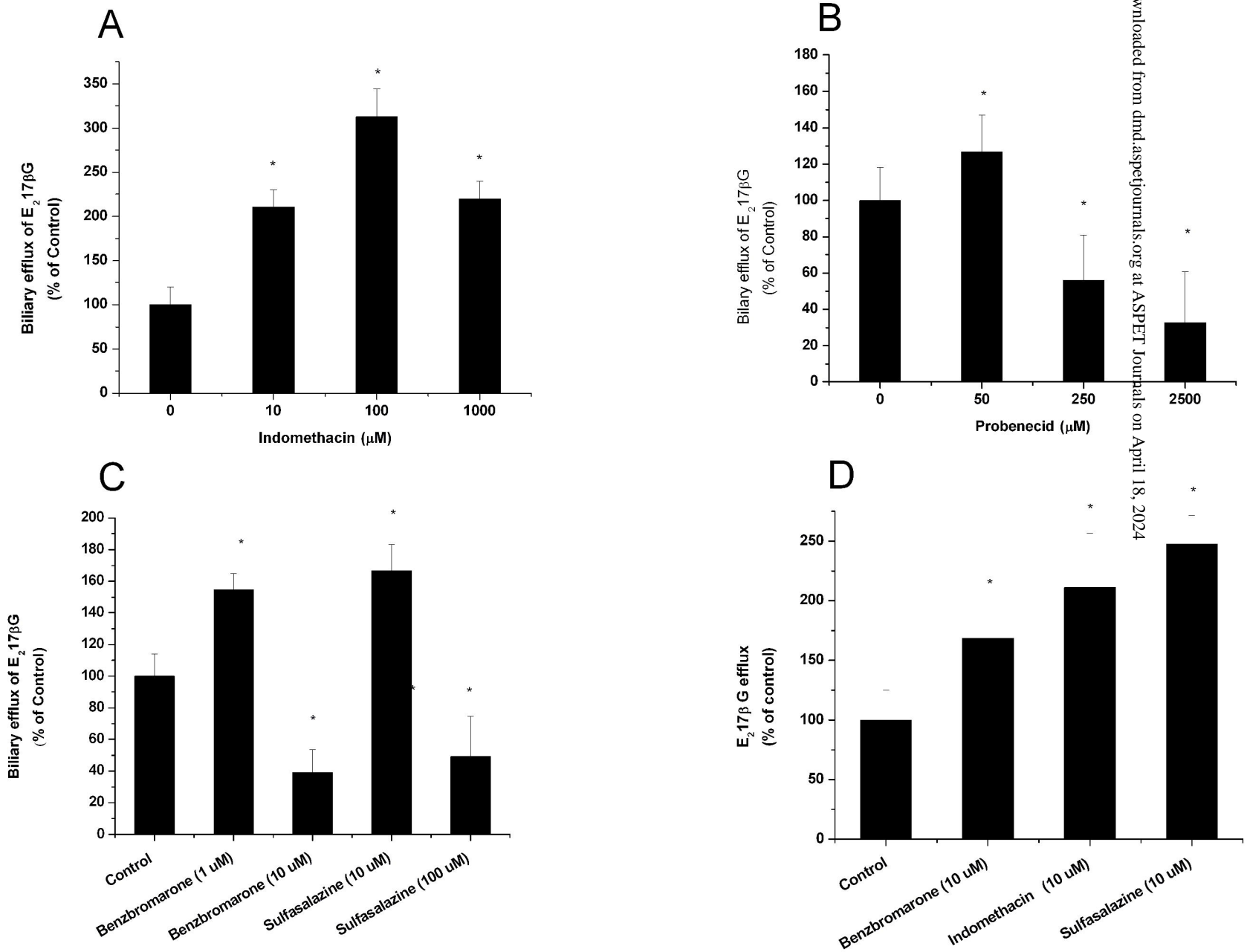


Figure 3



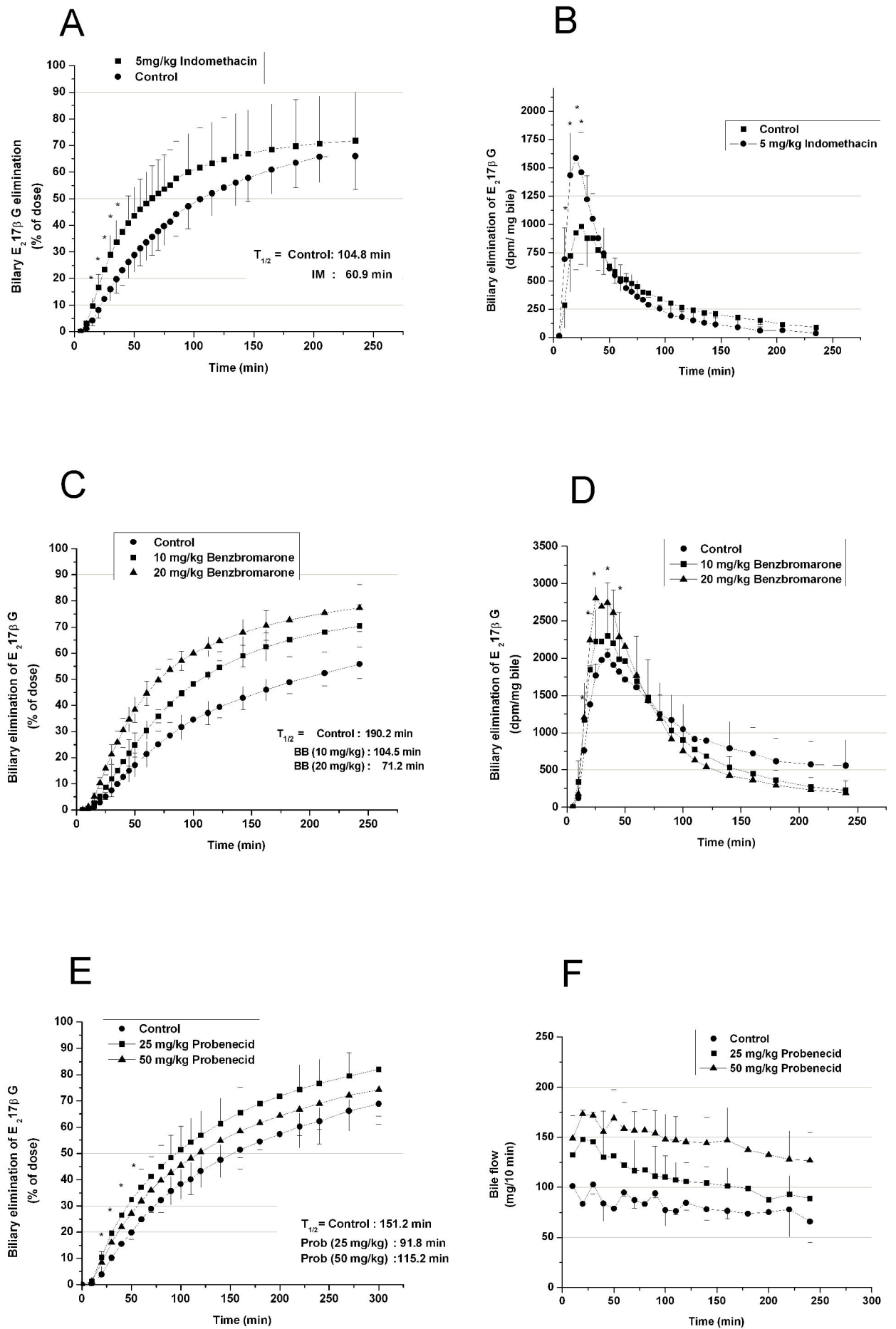


Figure 4