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1. **Title page:**

MRP2-mediated $E_2$17βG transport potentiation: *in vitro – in vivo* correlation and species specificity*

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2. **Running title page:** Potentiation of MRP2-mediated $E_2$17bG transport

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List of non-standard abbreviations:

$E_2$17βG : Estradiol-17-β-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-β-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 non-substrates) 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.

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) it's 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_2$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_2$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_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, 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 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_2$17βG (Huisman, 2005; Zimmermann, 2008). However, the physiological relevance 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 (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}H-Estradiol-17-β-D glucuronide ($E_2$17βG) 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 Enymology, 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_2III-5 (Alexis Corp., 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 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 μM pore size (Millipore, Billerica, MA, USA). Filters were washed with 200 μl 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 Ca^{2+} free Earle's balanced salt solution (EBSS) containing EGTA, then with the same buffer without chelating agent and finally with EBSS containing Ca^{2+} and type IV collagenase (Sigma-Aldrich, St. Louis, MO). Perfusions were carried out at 37^0 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 2×10^6 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, 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). Briefly, 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 ^{0}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 in order 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, 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}H-$E_2$17βG in 300 µl of saline was coadministered with the modulator compounds i.p. Control rats received the ^{3}H-$E_2$17βG 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 $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, 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 μM 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):

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

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_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 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 ~ 150 μM, while in the presence of 100 μM indomethacin the saturation curve of human MRP2 becomes a Michaelis-Menten-type hyperbolic one, 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 μM to 26.3 μM in the presence of 100 μM indomethacin, while 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 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 μM).

Table 1 summarizes 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 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 in case of the human protein compared to the rat one (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. 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_2$17βG at all concentrations (Fig.3/A). Probenecid (3/B) and

benzbromarone, sulfasalazine (3/C) potentiated the transport at low concentrations (50 μM, 1 μM, 10 μM, respectively), however, at higher concentrations (250 μM and 2500 μM; 10 μM; 100 μM; respectively) inhibited the biliary transport of $E_2$17βG. Similarly, in human hepatocyte cultures benzbromarone (10 μM), indomethacin (10 μM) and sulfasalazine (10 μM) potentiated the biliary efflux of $E_2$17βG (Fig.3/D).

***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% (Figure 4/A). Indomethacin significantly increased the biliary efflux of $E_2$17βG shortly after administration, as shown on Figure 4/B.

Benzbromarone also increased the biliary elimination of $E_2$17βG (Figure 4/C). 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. 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 $E_2$17βG 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 choleretic 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_2$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_2$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_2$17βG transport (reviewed in Borst, 2006b). Single digit K_m values were reported by a study (Cui, 1999) with Michaelis-Menten type kinetics for the human protein. The study by Zelcer *et al* (2003) similarly to our data has found a cooperative interaction with lower affinities ($K_{0.5}$ = 120 μM). The study utilizing human CMV showed Michaelis-Menten type kinetics with a K_m of 364 μM (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_2$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 μM) 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_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. 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 μM indomethacin, the K_m value decreases from 61.5 μM to 26.3 μM, 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_2$17βG 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_2$17βG. At 1 μM $E_2$17βG potentiation, while at higher drug concentrations inhibition is seen (Fig. 2). At 50 μM concentration of $E_2$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_2$17βG concentrations the site used for the heterotropic effect is available for the modulator drugs at lower concentration. 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 at lower drug concentration, which suggests that at this respective substrate – modulator concentrations the modulator competes efficiently with $E_2$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_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, 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 dugs. 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_2$17βG transport in 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 1987; Shitara 2003). Using another approach, Sasaki et al. (Sasaki, 2004) correlated the *in vivo* biliary clearance and *in vitro* transcellular transport of $E_2$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_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.

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10. Footnotes

[*]Financial support

[1]Equally contributed to the study

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11. Legends for figures.

Figure 1. Effect of indomethacin on the transport of $E_2$17βG by human MRP2 (A, B) and rat Mrp2 (C, D). In case of human MRP2 in the absence of 100 μM indomethacin (closed squares) the estimated apparent half-maximal rate ($K_{0.5}$) was 150 μM, while in the presence of indomethacin (closed triangles), 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 (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_2$17βG transport by rat Mrp2 at lower substrate concentrations (Km = 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_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 (gray squares) and 50 μM E217βG (black triangles). B) Effect of modulators on the transport of E217βG by rat Mrp2. The effect is investigated in the presence of 1 μM E217βG (gray squares) and 50 μM E217βG (black triangles).

Figure 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 (3A-C) or human (3D) hepatocytes. Experiments were performed at1 μM $E_2$17βG concentration. Data are expressed as % of control, means ± S.D. (n = 3). All experiments were performed with hepatocytes from 3 independent preparations. Asterisks (*) indicate significant difference compared to control ($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_2$17βG studied *in vivo* using rats. Each point represents the mean value ± S.D., for n = 3. Asterisks (*) indicate significant difference compared to control ($p < 0.05$, determined by Student's test).

Table 1 Concentration of modulators (μM) causing maximal potentiation (%) of $E_2$17βG transport in the assays used

	MRP2 VT	rat Mrp2 VT	MRP2 sandwich	rat Mrp2 sandwich
	Modulator concentration (μM) / 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

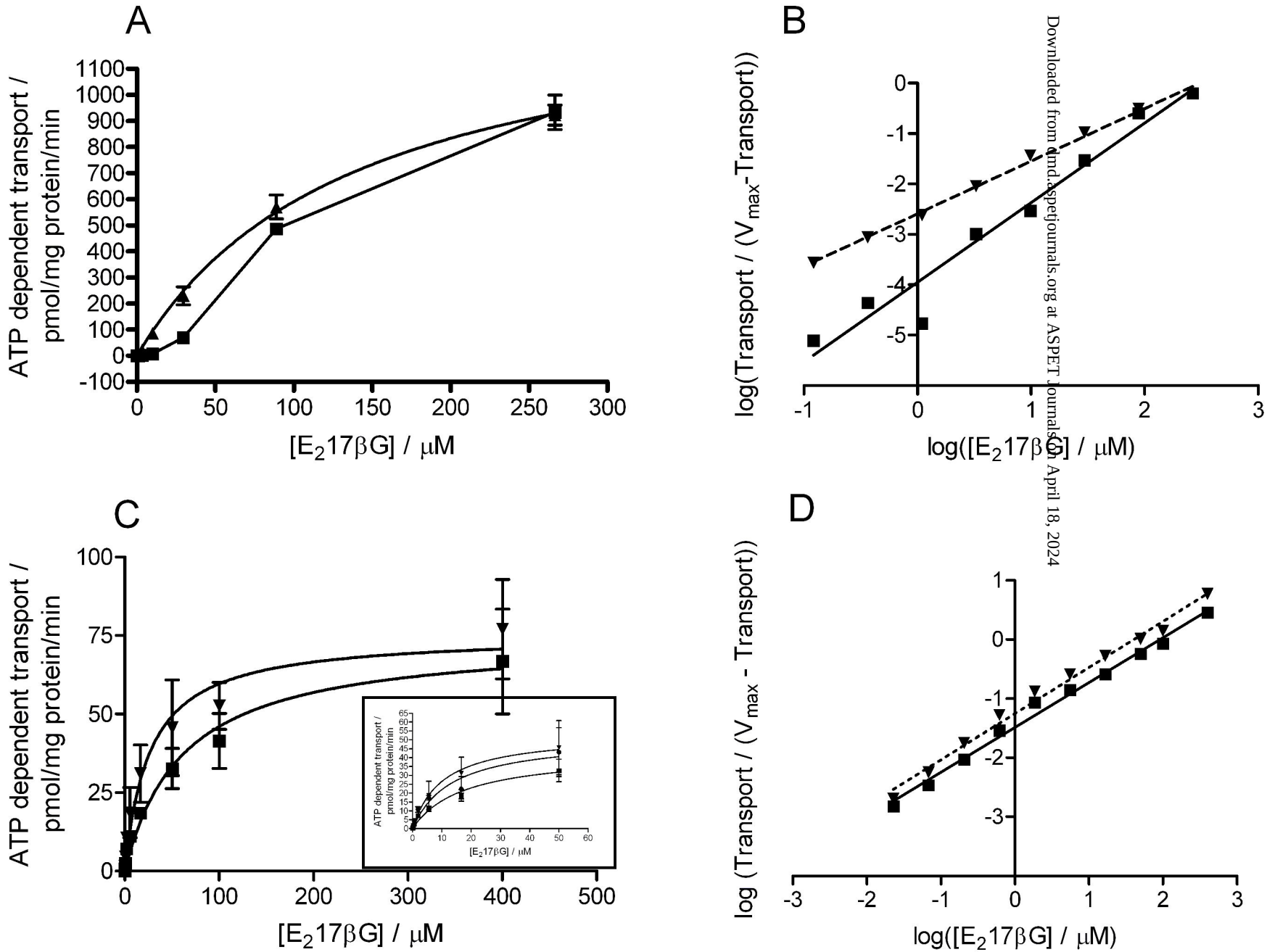
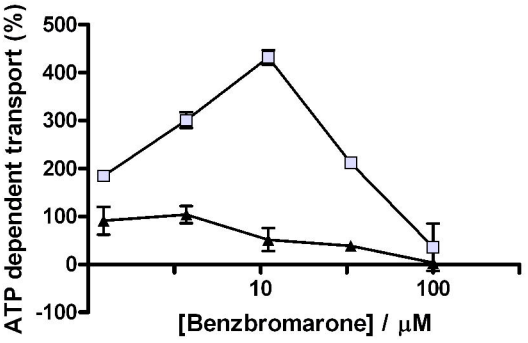
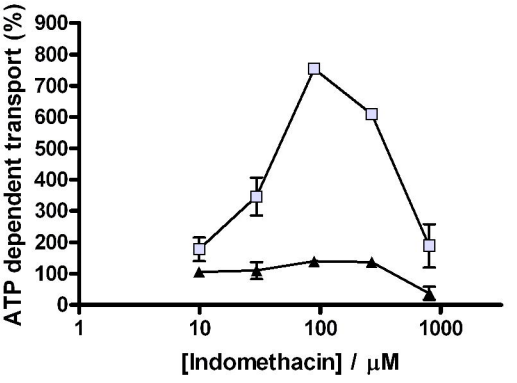
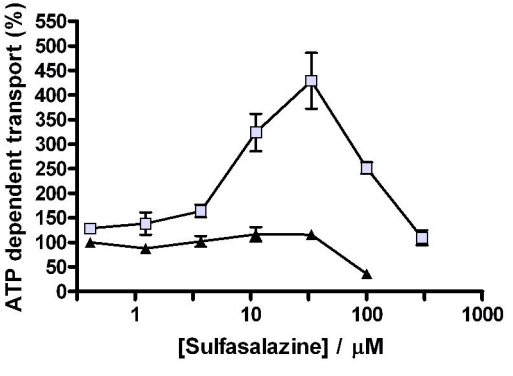


Figure 1.


ATP dependent transport (%)
500
400
300
200
100
0
-100
10
100
[Benzbromarone] / μM


ATP dependent transport (%)
900
800
700
600
500
400
300
200
100
0
1
10
100
1000
[Indomethacin] / μM


ATP dependent transport (%)
550
500
450
400
350
300
250
200
150
100
50
0
1
10
100
1000
[Sulfasalazine] / μM

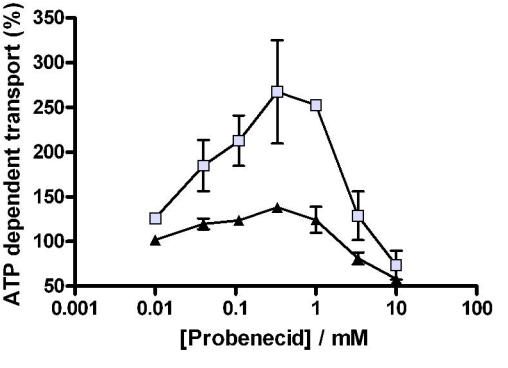
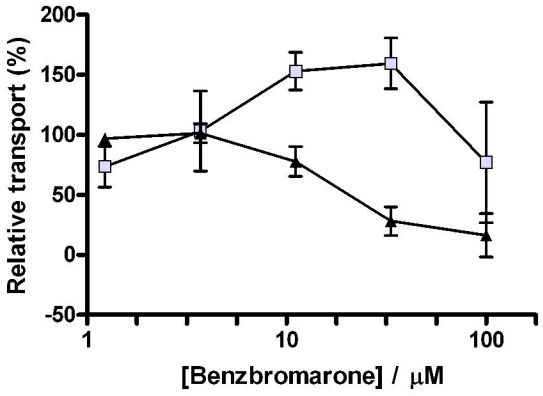
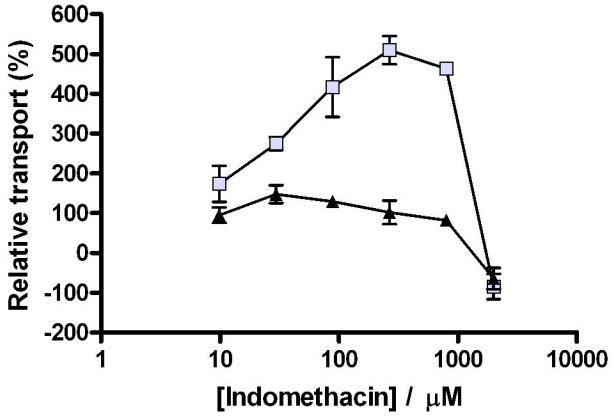
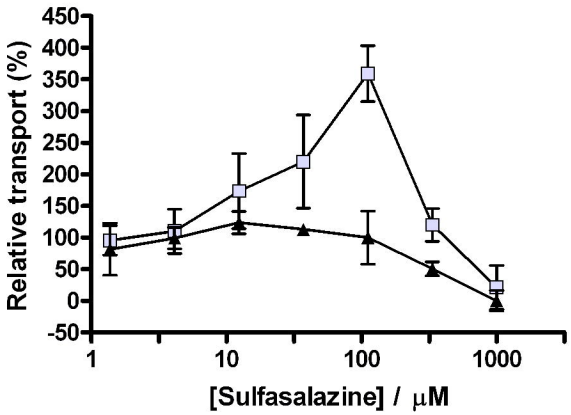

ATP dependent transport (%)
350
300
250
200
150
100
50
0.001
0.01
0.1
1
10
100
[Probenecid] / mM

Figure 2/A


Relative transport (%)
200
150
100
50
0
-50
1
10
100
[Benzbromarone] / μM


Relative transport (%)
600
500
400
300
200
100
0
-100
-200
1
10
100
1000
10000
[Indomethacin] / μM


Relative transport (%)
450
400
350
300
250
200
150
100
50
0
-50
1
10
100
1000
[Sulfasalazine] / μM

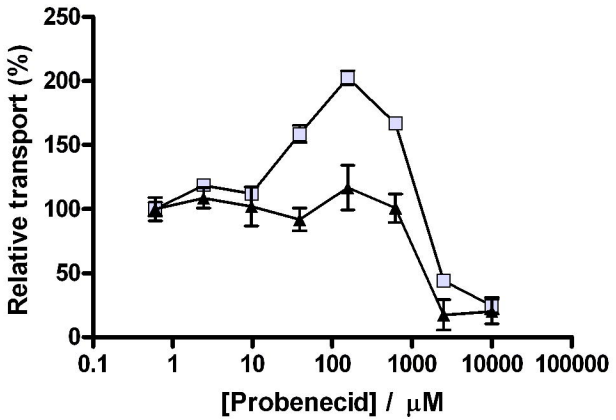
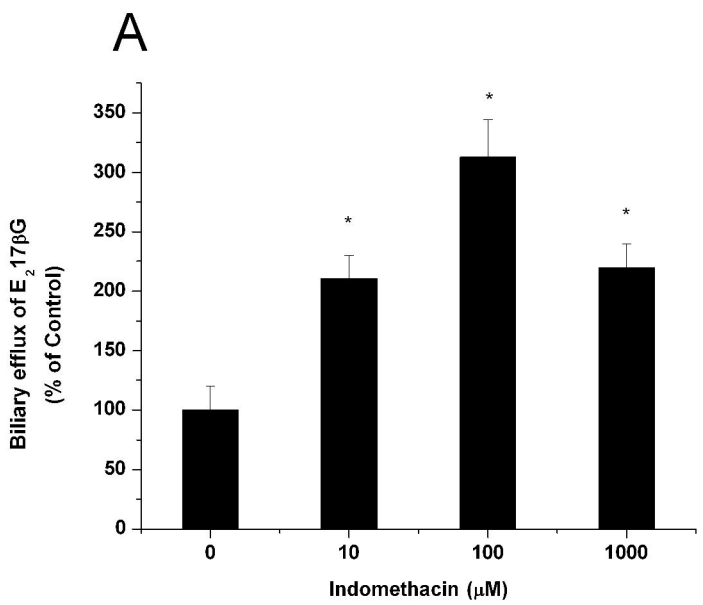
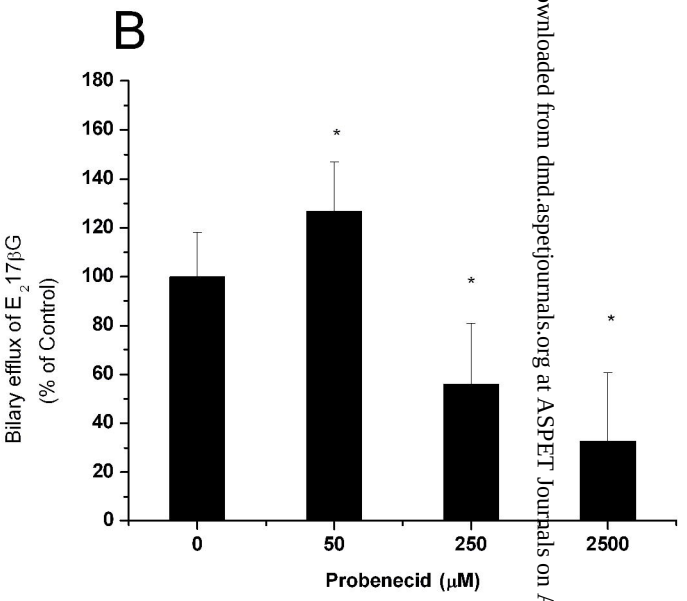
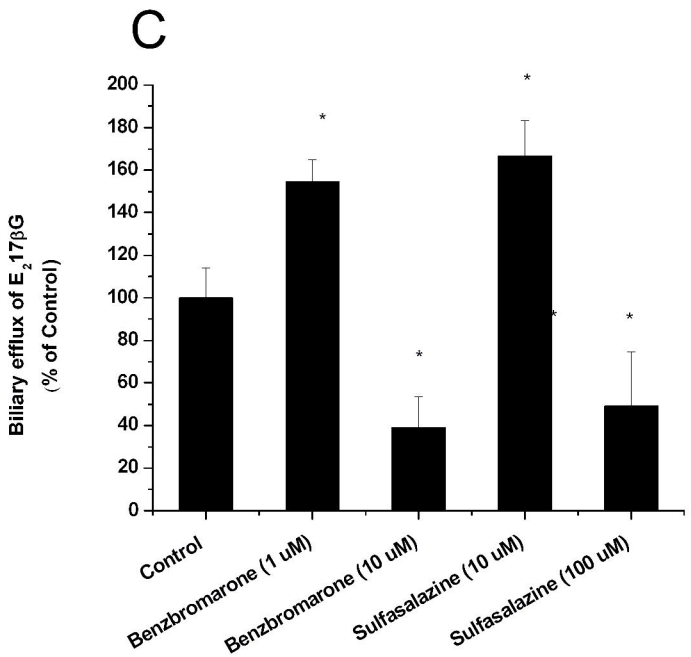

Relative transport (%)
250
200
150
100
50
0
0.1
1
10
100
1000
10000
100000
[Probenecid] / μM

Figure 2/B







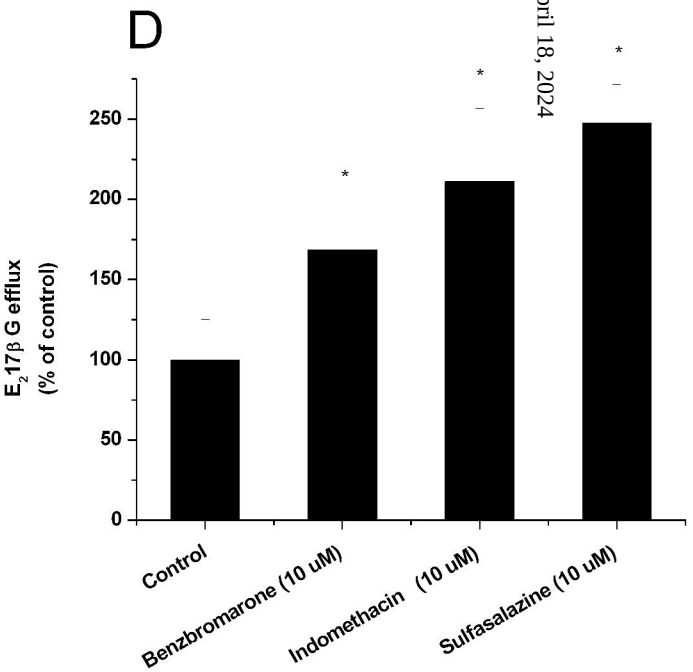


Figure 3

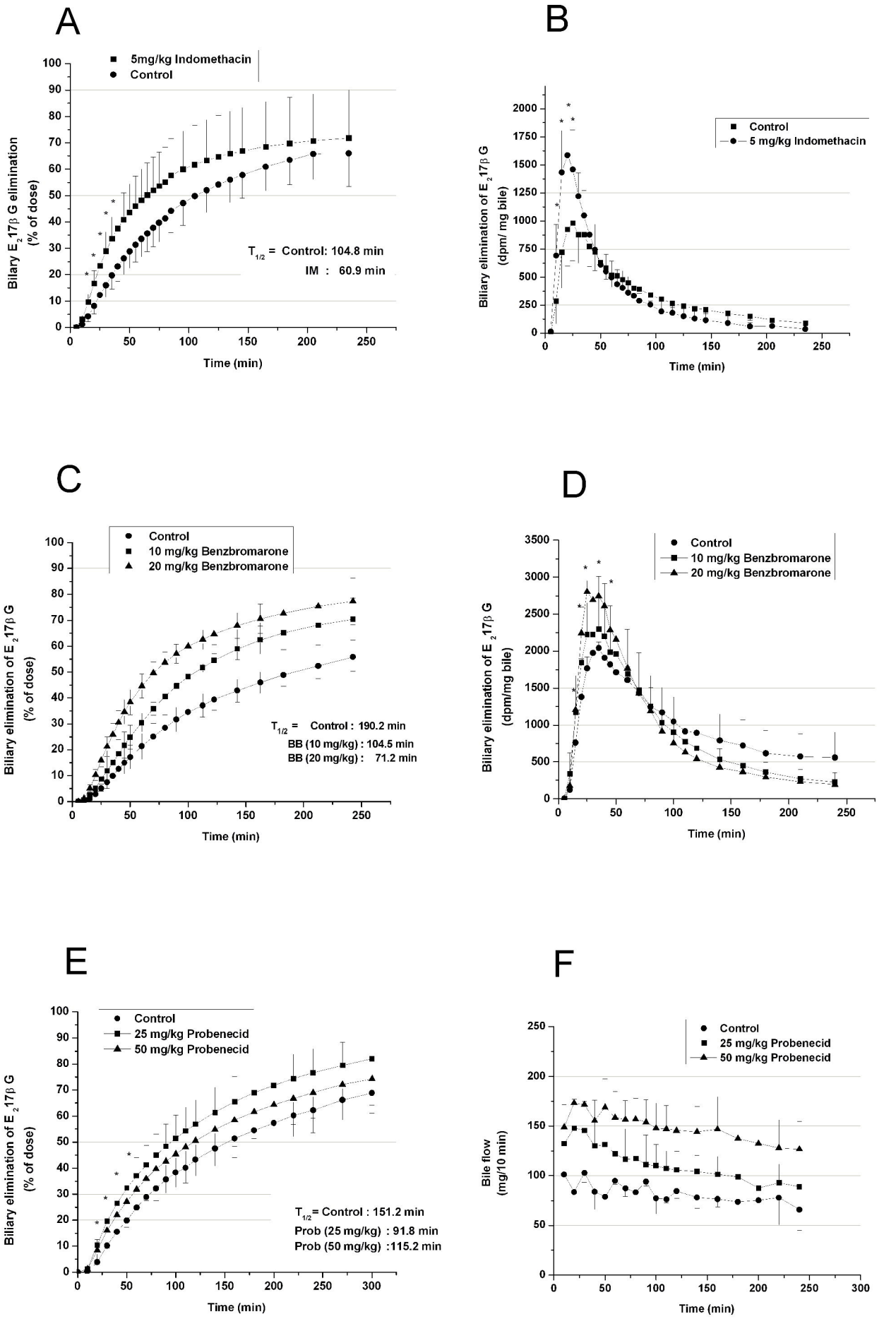

A
5mg/kg Indomethacin
Control
Bilary $E_2$17β G elimination (% of dose)
$T_{1/2}$ = Control: 104.8 min
IM : 60.9 min
Time (min)
B
Control
5 mg/kg Indomethacin
Biliary elimination of $E_2$17β G (dpm/ mg bile)
Time (min)
C
Control
10 mg/kg Benzbromarone
20 mg/kg Benzbromarone
Biliary elimination of $E_2$17β G (% of dose)
$T_{1/2}$ = Control : 190.2 min
BB (10 mg/kg) : 104.5 min
BB (20 mg/kg) : 71.2 min
Time (min)
D
Control
10 mg/kg Benzbromarone
20 mg/kg Benzbromarone
Biliary elimination of $E_2$17β G (dpm/mg bile)
Time (min)
E
Control
25 mg/kg Probenecid
50 mg/kg Probenecid
Biliary elimination of $E_2$17β G (% of dose)
$T_{1/2}$ = Control : 151.2 min
Prob (25 mg/kg) : 91.8 min
Prob (50 mg/kg) :115.2 min
Time (min)
F
Control
25 mg/kg Probenecid
50 mg/kg Probenecid
Bile flow (mg/10 min)
Time (min)

Figure 4