Transport of ethinylestradiol glucuronide and ethinylestradiol sulfate by the multidrug resistance proteins MRP1, MRP2, and MRP3

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Running title

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The document statistics

Text pages: 17 pages;

Figures: 9 pages;

References: 6 pages;

Abstract: 230 words;

Introduction: 750 words;

Discussion: 1500 words.

Non-standard abbreviations

MRP1, multidrug resistance protein 1; MRP2, multidrug resistance protein 2; MRP3, multidrug resistance protein 3; Sf9, *Spodoptera frugiperda*; $E_2 17\beta G$, 17β-estradiol-17β-D-glucuronide; EA-SG, ethacrynic acid glutathione; EE-G, 3-O-glucuronide of EE; EE-S, 3-O-Sulfate of EE; EE, ethinylestradiol.

The recommended section

Absorption, Distribution, Metabolism, & Excretion

JPET #62091

Abstract

Ethinylestradiol (EE) is one of the key constituents of oral contraceptives. Major metabolites of EE in humans are the glucuronide and sulfate conjugates, EE-3-O-glucuronide (EE-G) and EE-3-O-sulfate (EE-S). In the present study, transport of EE-G and EE-S by the human multidrug resistance proteins MRP1, MRP2 and MRP3 was investigated using inside-out membrane vesicles, isolated from Sf9 cells expressing human MRP1, MRP2, or MRP3. Vesicular uptake studies showed that EE-G was not a substrate for MRP1, while an ATP-dependent and saturable transport of [^{3}H] EE-G was observed in MRP2 (K_m 35.1 ± 3.5 µM) and MRP3 (K_m 9.2 ± 2.3 µM) containing vesicles. EE-S was not transported by either MRP1, MRP2 or MRP3. However, low concentrations of EE-S stimulated MRP2-mediated uptake of ethacrynic acid glutathione (EA-SG). EE-S also stimulated MRP2 and MRP3-mediated uptake of 17β-estradiol-17β-D-glucuronide (E$_2$17βG). Interestingly, EE-S stimulated strongly MRP2- and MRP3-mediated uptake of EE-G by increasing its apparent transport affinity, while no reciprocal stimulation of EE-S uptake by EE-G was observed. These data indicate that EE-S allosterically stimulates MRP2 and MRP3-mediated transport of EE-G, and is not co-transported with EE-G. Our studies demonstrate specific active transport of a pharmacologically relevant drug conjugate by human MRP2 and MRP3, involving complex interactions with other organic anions. We also suggest that caution needs to be taken when using only competition studies as screening tools to identify substrates or inhibitors of MRP-mediated transport.

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17α-ethinyl estradiol (EE), a synthetic estrogen, is an essential constituent of oral contraceptives (OC), which have been widely prescribed since the 1970s. Over 60 million women currently take OC and their safety profile is well established. Numerous examples are known (Stockley, 1999) where co-administration of EE with a range of other drugs can result in decreased plasma levels of EE with the commensurate failure of contraception and breakthrough bleedings. Alterations in EE metabolism and disposition are proposed to occur via induction of hepatic or gut enzymes involved in the metabolism and/or transport of EE and its metabolites.

The pharmacokinetics and metabolism of EE in humans occurs both in gut and liver, respectively, where the mean bioavailability is reported to be 45% (Rogers et al., 1987; Back et al., 1982). EE mainly undergoes sulfation and glucuronidation resulting in the formation of EE-3-O-sulfate (EE-S) and EE-3-O-glucuronide (EE-G). EE-G and EE-S have been detected in bile, intestinal mucosa, and urine (Maggs et al., 1983; Pacifici et al., 1988; Orme et al., 1989). However, the mechanisms involved in the transport and elimination of these metabolites are unknown.

Multidrug resistance proteins (MRPs), transporters belonging to the ATP-binding cassette (ABC) superfamily, have been suggested to play an important role in the transport and detoxification of a wide range of endogenous compounds and xenobiotics (Borst et al., 2000, 2002). The MRP family consists of nine members, which are referred to as MRP1-9 (Borst and Oude Elferink, 2002). MRP1 (ABCC1) is localized in the basolateral membranes of polarized cells (Evers et al., 1996; Cole et al., 1998) and is expressed in all tissues except the liver (Zaman et al, 1994). In contrast, MRP2 (ABCC2) is expressed in the canalicular membrane of hepatocytes, the apical membrane of the small intestine, the apical membrane of the proximal tubules of the kidney, and placental trophoblasts (Paulusma et al., 1997; Schaub et al., 1997, 1999; St-Pierre et al., 2000).

MRP2 therefore may be involved in hepatobiliary-, renal- and intestinal excretion of compounds, and protection of the fetus. MRP3 (ABCC3) is localized in the basolateral membranes of polarized cells (Kőnig et al., 1999). It is expressed in the gut, pancreas, liver cholangiocytes, adrenals, and kidney, and is highly induced in hepatocytes under cholestatic condition (Scheffer et al., 2002; Donner et al., 2001). MRP3 may play a role in enterocytes by transporting compounds from the intestine into the blood stream, and under cholestatic conditions by removing toxic organic anions from hepatocytes (Keppler et al., 2000).

MRP1-3 are able to transport conjugated and non-conjugated organic anions (Borst et al., 2000, 2002). The substrate specificity of MRP1 and MRP2 is overlapping, and includes glutathione (GSH), glucuronide and sulfate conjugates, some non-conjugated organic anions, and various neutral or positively charged drugs (Cole et al., 1994; Cui et al., 1999). MRP3 also transports organic anions but prefers glucuronide and sulfate conjugates over glutathione conjugates (Hirohashi et al., 1999, 2000; Zelcer et al., 2003a).

MRP1-3 have been shown to interact with a wide range of substrates, including several physiologically or pharmacologically active organic anions. For instance, GSH plays an important role in MRP1 and MRP2-mediated transport of a number of compounds. In some cases compounds are co-transported with GSH but in others not (Loe et al., 1998; Renes et al., 1999; Evers et al., 2000; Qian et al., 2001; Leslie et al., 2001). Bodo et al. (2003) showed that some organic anions, including indomethacin, furosemide, probenecid, and some bile acids, significantly stimulated MRP2-mediated $E_2 17\beta G$ uptake, but inhibited its uptake by MRP3. In MDCKII-MRP2 cells, probenecid enhanced vectorial transport of the HIV protease inhibitors saquinavir, ritonavir, and indinavir (Huisman et al., 2002). So far, the mechanism explaining these complex stimulation and inhibition effects has not been elucidated but they suggest that MRP1-3

have multiple drug binding sites (Loe et al., 1998; Daoud et al., 2000; Borst and Oude Elferink, 2002; Deeley et al., 2003).

Here, we investigate the ability of MRP1-3 to transport EE-G and EE-S using membrane vesicles isolated from baculovirus infected *Spodoptera frugiperda* (Sf9) insect cells expressing MRP1, MRP2 or MRP3. While we determined that EE-G was a good substrate for MRP2 and MRP3, neither MRP1, MRP2 nor MRP3 were capable of transporting EE-S. Interestingly, EE-S stimulated strongly MRP2 and MRP3-mediated transport of EE-G and had variable effects on the transport of other substrates. The interpretation of our findings in light of models explaining MRP-mediated transport is discussed. In addition, our data suggest that MRP2 and MRP3 are the relevant transporters *in vivo* for the export of EE-G from intestine and liver.

Materials and Methods

Materials

[^{3}H] EE-S (10.09 µCi/nmol) and [^{3}H] EE-G (10.09 µCi/nmol) were synthesized by the Department of Drug Metabolism, Merck Research Laboratories, Rahway, NJ (detailed methods available upon request). The purity of [^{3}H] EE-S and [^{3}H] EE-G was verified by HPLC (>99.9%). [^{14}C] Ethacrynic acid glutathione conjugate (EA-SG) (65mCi/mmol) was synthesized by the Merck Labeled Compound Synthesis Group, Rahway, NJ by reacting [^{14}C] ethacrynic acid with glutathione as previously described (Ploemen et al., 1990). [^{14}C] Ethacrynic acid was obtained by substituting [^{14}C] paraformaldehyde into the method described in US Patent 3,255,241 for preparation of unlabeled material. The purity of [^{14}C] EA-SG verified by HPLC was 98%. [^{3}H] Estradiol-17β-D-glucuronide (E$_2$17βG) (40.5 Ci/mmol) was obtained from PerkinElmer life Sciences Inc (Boston, MA). EE-G and EE-S was obtained from Steraloids (Newport, RI). E$_2$17βG, creatine phosphate and creatine phosphokinase were purchased from Sigma (St. Louis, MO). All chemicals were of the highest analytical purity grade.

Preparation of membrane vesicles

Recombinant baculovirus containing MRP1, MRP2 cDNAs and β-galactosidase gene (control) were obtained from Solvo Biotechnology (Szeged, Hungary). Baculovirus containing MRP3 cDNA was kindly provided by Dr. Piet Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). *Spodoptera frugiperda* (Sf9) cells in suspension were grown in Sf-900 II SFM medium in the absence of serum (Invitrogen, Carlsbad, CA). About 4 x 10^7 Sf9 cells were seeded in 175 cm^2 tissue culture flasks. After the cells became attached, the medium was removed and 3 mL of medium and 3 mL of virus stock containing MRP1, MRP2 or MRP3 (about 5-8 x 10^7 virus/mL) was added to infect the cells. One hour after addition of the virus to the cells, cell culture medium was added up to a final volume of 30 mL. After incubation for 72 hr at 26°C, the

cells were harvested and washed twice in ice-cold washing buffer (50 mM Tris/HCl, 300 mM Mannitol, 0.5 mM PMSF, pH 7.0) and centrifuged at 800 x g for 5 min at 4°C. The cell pellet was resuspended in ice-cold TMEP buffer (50 mM Tris, 50 mM Mannitol, 2 mM EGTA-Tris, 2 mM DTT, Aprotinin (8 µg/mL), Leupeptin (10 µg/mL), PMSF (50 µg/mL), pH 7.0) and homogenized for 10 min on ice using a tight-fitting Dounce homogenizer. After centrifugation at 800 x g for 10 min at 4 °C, the supernatant was collected and centrifuged at 100,000 x g for 1 hr at 4 °C. The pellet was resuspended in TMEP buffer and passed 20 times through a 27-gauge needle. The vesicles were dispensed in aliquots, frozen in liquid nitrogen, and stored at –80 °C until use.

Vesicular uptake studies

Vesicular uptake studies were performed using the rapid filtration technique as reported previously (Chu et al., 1997). The transport medium contained the radiolabeled ligand, 250 mM sucrose, 10 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 5 mM adenosine 5' triphosphate (ATP), or 5 mM adenosine monophosphate (AMP), and an ATP-regenerating system (10 mM creatine phosphate and 100 µg/mL creatine phosphokinase). The uptake study was performed at 37 °C. After preincubation for 3 min at 37 °C, the uptake study was started by the addition of vesicle suspension (10 µg protein). The final incubation volume was 20 µl. In the inhibition study, the inhibitors were dissolved in transport buffer and preincubated with ligands for 3 min. At designated time points, transport was terminated by adding 1 mL of ice-cold stop solution containing 10 mM Tris/HCl (pH 7.4), 250 mM sucrose, and 100 mM NaCl. The stopped reaction mixture was filtered through 0.45 µm HA Millipore filters (Millipore Corporation, Bedford, MA) and subsequently washed twice with 5 mL ice-cold stop solution. For the uptake study with EE-S, glass fiber (type A/E) filters (Gelman Sciences, Dorval, Quebec, Canada) were used to minimize non-specific binding to membrane filters. The

radioactivity retained on the filter and in the reaction mixture was measured in a liquid scintillation counter (LS 6000 Beckman instruments, Fullerton, CA). ATP-dependent uptake was determined as the difference in uptake in the presence and absence of ATP.

Western blot analysis

Membrane vesicles isolated from baculovirus infected Sf9 cells were solubilized in Laemli sample buffer (Bio-Rad, Hercules, CA) containing 2.5% of β-mercaptoethanol and separated in a 7.5% denaturing polyacrylamide gel. Gels were immuno-blotted onto nitrocellulose membranes. MRP1, 2 and 3 were detected with the specific monoclonal antibodies MRP-R1 (1:500), M_2I4 (1:500), and $MRP-III_{21}$ (1:40), respectively, as previously described (Bakos et al., 2000). The blots were then developed with the enhanced chemiluminescence (ECL) kit (Amersham Corp., Arlington Heights, IL) and exposed to ECL hyperfilm (Amersham Corp. Arlington Heights, IL).

Data analysis

Kinetic parameters for the ATP-dependent uptake were obtained by fitting the data to the following equation:

$$V_o = V_{max} \times S / (K_m + S) \qquad \text{Eq. (1)}$$

where V_o is the initial uptake rate of substrate (pmol/min/mg protein), S is the substrate concentration in the medium (μM), K_m is the Michaelis constant (μM) and V_{max} is the maximum uptake rate (pmol/min/mg protein). To obtain estimates of kinetic parameters, the uptake data were fitted to Eq. (1) by a nonlinear least-squares method using *KaleidaGraph* (Synergy Software, Reading PA).

The inhibition constant (K_i) values for evaluating the inhibitory effect of EE-G on the uptake of [³H] EA-SG by MRP2 and [³H] $E_2$17βG by MRP3 were obtained by fitting the following equation to the data as described before (Chu et al., 1997):

$$V_{(+I)} / V_{(-I)} = 1 / [1 + (I / K_i)] \qquad \text{Eq (2)}$$

$V_{(+I)}$ and $V_{(-I)}$ represent the transport velocity in the presence and absence of inhibitor, respectively, and I is the inhibitor concentration. This equation was derived based on the assumptions that firstly inhibition was competitive or noncompetitive and secondly that the [^{3}H] EA-SG concentration (2 µM) used was much lower than the K_m value (15 µM for MRP2, data not shown), and that the [^{3}H] $E_2$17βG concentration (0.4 µM) used was much lower than the K_m value (27 µM for MRP3, data not shown).

Results

Detection of MRP1, MRP2 and MRP3 in membrane vesicles

By Western blotting, MRP1, MRP2 and MRP3 were detected with specific antibodies in membrane vesicles isolated from baculovirus infected Sf9 cells expressing cDNAs encoding MRP1, MRP2 or MRP3, respectively. As shown in Fig. 1 (panels A-C), MRP1 (lanes 3, 4), MRP2 (lanes 5, 6), and MRP3 (lanes 7, 8) were detectable in these vesicles. Levels were similar as in vesicles used by Bodo et al. (2003; data not shown). No signal was detected in vesicles isolated from control β-galactosidase expressing cells (lanes 1, 2). Because MRP1, MRP2, and MRP3 were detected using different monoclonal antibodies, it was not possible to compare the exact expression levels of the proteins in these membrane vesicles. Bodo et al. (2003), however, detected comparable amounts of MRP1-3 in Sf9 membrane vesicles after staining gels with Coomassie Brilliant Blue. Since we do not know what percentage of MRP was transport competent, the absolute values of the transport measured by different transporters should be compared with care. As a positive control for the quality of the vesicles, functional activity of MRP1, MRP2, and MRP3 was confirmed by ATP-dependent uptake of EA-SG and $E_2 17\beta G$ (data not shown).

Transport of EE-G by MRP1, MRP2 and MRP3

To evaluate whether EE-G was a substrate for MRP1, MRP2 or MRP3, uptake of [^{3}H] EE-G (0.2 µM) into membrane vesicles containing MRP1, MRP2 and MRP3 was investigated (Fig. 2). ATP-dependent uptake of [^{3}H] EE-G (0.2 µM) in MRP1 containing vesicles was low and comparable to that observed in control Sf9 membrane vesicles (Fig. 2A). In contrast, ATP-dependent uptake of [^{3}H] EE-G (0.2 µM) was much higher in MRP2 containing vesicles and showed linearity within 5 min (Fig. 2B). As shown in Fig 2C, significant time- and ATP-dependent uptake of EE-G was also observed in MRP3

containing vesicles. Several studies have shown that MRP1-mediated transport of some compounds, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol glucuronide (NNAL-O-glucuronide) and estrone-3-sulfate, is GSH dependent (Loe et al., 1998; Sakamoto et al., 1999; Qian et al., 2001; Leslie et al., 2001). We therefore evaluated whether GSH stimulates MRP1-mediated EE-G uptake. In this experiment, 10mM dithiothreitol (DTT) was added to the reaction mixture as a reducing agent (Leslie et al., 2001). Our results, however, indicated that 3 mM GSH did not have an effect on MRP1-mediated EE-G uptake (data not shown).

Concentration dependence of ATP-dependent uptake of EE-G was studied in MRP2 and MRP3 containing membrane vesicles. Since the uptake of [^{3}H] EE-G (0.2 µM) in the presence of 5 mM ATP was linear for up to 5 min (Fig 2B, C), the initial rate for the uptake of EE-G at various substrate concentrations was determined after a 5 min incubation. Kinetic analysis revealed that the ATP-dependent uptake of EE-G by MRP2 and MRP3 containing vesicles was saturable with a K_m of 35 $\pm$ 3.5 µM and a V_{max} of 310 $\pm$10 pmol/min/mg protein for MRP2-mediated uptake (Fig. 3A), and a K_m of 9.2 $\pm$ 2.3 µM and a V_{max} of 58 $\pm$ 4.2 pmol/min/mg protein for MRP3-mediated uptake (Fig. 3B).

Transport of EE-S by MRP1, MRP2 and MRP3

To evaluate whether EE-S was a substrate for MRP1, MRP2 or MRP3, uptake of [^{3}H] EE-S (0.2 µM) was investigated in MRP1, MRP2 and MRP3 containing vesicles, and control Sf9 membrane vesicles containing β-galactosidase. No ATP-dependent uptake of EE-S higher than in the control vesicles was observed by any of these transporters (Fig 4A-C). Our studies also showed that GSH up to 3 mM did not stimulate MRP1, MRP2 and MRP3-mediated EE-S uptake (data not shown). Studies using membrane vesicles isolated from MDCKII-MRP2 cells also indicated that EE-S was not a substrate of MRP2 (unpublished observation).

Effect of EE-G and EE-S on MRP2 and MRP3-mediated transport by EA-SG and E$_2$17βG

Recently, it has been shown that compounds can have both an inhibitory and/or stimulatory effect on MRP-mediated transport both in intact cells and in membrane vesicles (Evers et al., 2000; Bakos et al., 2000; Qian et al., 2001; Huisman et al., 2002). We therefore investigated the effect of EE-G and EE-S on MRP2 and MRP3 mediated uptake by EA-SG and E$_2$17βG, known substrates for these transporters (Evers et al., 1998; Hirohashi et al., 1999). Uptake of EA-SG and E$_2$17βG by MRP2 and MRP3, respectively, was ATP-dependent and saturable. The K$_m$ value for EA-SG uptake by MRP2 was 15 μM and the K$_m$ value for E$_2$17βG uptake by MRP3 was 27 μM (data not shown). Recent studies showed that the rate of E$_2$17βG transport by MRP2 increased sigmoidally with half-maximal transport of 120 μM (Zelcer et al., 2003b, Bodo et al., 2003). EE-G significantly inhibited ATP-dependent uptake of EA-SG in MRP2 containing vesicles with a K$_i$ value of 20 ± 1.1 μM (Fig 5A). EE-G also significantly inhibited MRP3-mediated uptake of E$_2$17βG in MRP3 expressing vesicles with a K$_i$ value of 5.0 ± 0.6 μM (Fig 5B). In contrast, EE-S at relatively low concentrations (2-20 μM) stimulated MRP2-mediated uptake of EA-SG up to 1.5-fold (Fig. 6A). EE-S at high concentration (100 μM), however, inhibited MRP2-mediated uptake of EA-SG by approximately 50% (Fig. 6A). Unlike its effect on EA-SG, EE-S only showed a strong stimulatory effect on MRP2-mediated E$_2$17βG uptake within the concentrations tested (2-100 μM) (Fig. 6B). EE-S at a concentration of 100 μM enhanced E$_2$17βG uptake up to 17-fold (Fig. 6B). EE-S at low concentration (2-20 μM) also showed a stimulatory effect (3.8-fold) on MRP3–mediated uptake of E$_2$17βG but slightly inhibited its uptake at a very high concentration (100 μM; Fig 6C).

Effect of EE-S on MRP1-3-mediated uptake of EE-G

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A possible stimulatory effect of EE-S on MRP1-3-mediated uptake of EE-G was also evaluated. EE-S at 2-100 μM had no stimulatory effect on ATP-dependent uptake of EE-G in MRP1 containing vesicles (Fig. 7A), but rather inhibited its low-level uptake. Since EE-G uptake by MRP1 was not higher than in control vesicles (Fig. 2A), this effect was possibly due to inhibition of a low endogenous MRP-like activity present in Sf9 cells. In contrast, EE-S (2-100 μM) stimulated MRP2-mediated ATP-dependent uptake of EE-G strongly (Fig. 7B). EE-S at a concentration of 20 μM increased the ATP-dependent initial uptake rate of EE-G 15-fold. Stimulation was further enhanced up to 22-fold in the presence of 100 μM EE-S, the highest concentration tested. EE-S (2-100 μM) also showed a significant stimulatory effect on MRP3-mediated uptake of EE-G (Fig. 7C). Strongest stimulation was observed at EE-S concentration of 20 μM (about 5-fold), while the stimulatory effect slowly decreased at higher concentrations (Fig. 7C). The effects of EE-S on MRP2 and MRP3-mediated uptake of EE-G were further characterized by determining the initial uptake rates of EE-G at various substrate concentrations in the presence of EE-S (20 μM; Fig. 8A and 8B, respectively). In the presence of EE-S (20 μM), the K_m for uptake of EE-G by MRP2 was calculated to be 4.1 ± 0.7 μM, which was about 9-fold lower than the K_m determined in the absence of EE-S (Fig. 3A, $K_m = 35 \pm 3.5$ μM). In the presence of EE-S, the V_{max} was 435 ± 16 pmol/min/mg protein, which was slightly higher than that in the absence of EE-S (Fig. 3A, $V_{max} = 310 \pm 10$ pmol/min/mg protein). For MRP3-mediated uptake of EE-G, in the presence of 20 μM EE-S, the K_m was estimated to be 0.5 ± 0.2 μM, which was about 18-fold lower than the K_m obtained in the absence of EE-S (Fig. 3B, $K_m = 9.2 \pm 2.3$ μM). The V_{max} value was 88 ± 5.6 pmol/min/mg protein, which was again slightly higher than that in the absence of EE-S (Fig. 3B, $V_{max} = 58 \pm 4.2$ pmol/min/mg protein).

Effect of EE-G on MRP2 and MRP3–mediated [³H] EE-S uptake

The stimulatory effect of EE-S on transport of EE-G could be explained by a mechanism in which both compounds are co-transported or by an allosteric effect of EE-S on MRP2 or MRP3 resulting in an increased transport of EE-G. To investigate whether transport of EE-S could be reciprocally stimulated by EE-G, the effect of EE-G on uptake of [^{3}H] EE-S (20 μM) by MRP2 and MRP3 was evaluated. As shown in Fig. 9, EE-G (0.5-100 μM) did not cause a significant stimulation of ATP-dependent uptake of EE-S by MRP2 (Fig.9A) or MRP3 (Fig. 9B) containing vesicles. The slight ATP-dependent uptake of EE-S observed in MRP2 containing vesicles at EE-G concentrations of 5, 20, and 100 μM was not significant, as this stimulatory effect could not be confirmed in a subsequent time course experiment. No significant ATP-dependent uptake of EE-S in the presence of various concentrations of EE-G was detected in these experiments (data not shown).

Discussion

In this study, we show that EE-G is a substrate of MRP2 and MRP3, but not of MRP1 (Fig 2 and 3). EE-S was not transported by MRP1-3 (Fig. 4), also not in the presence of GSH (data not shown). Since EE undergoes extensive gut (E_g 44%) and liver (E_h 25%) first-pass metabolism in humans (Back et al., 1982), MRP2 and MRP3 may be the physiologically relevant transporters for the export of EE-G from these organs. These transporters do not transport EE-S, however. Furthermore, we find a strong allosteric stimulation between two pharmacologically relevant conjugated metabolites of EE, EE-G and EE-S. The stimulatory effect of EE-S on MRP2 and MRP3-mediated transport of EE-G (Fig. 7) might be relevant *in vivo*, and therefore, could potentially have an impact on the *in vivo* disposition of EE and its interaction with other drugs.

Interestingly, EE-S showed a pronounced effect on MRP2 and MRP3-mediated uptake of EE-G, but the extent of this interaction varied among these transporters. EE-S stimulated MRP2-mediated uptake of EE-G strongly (Fig. 7B). Uptake was enhanced up to 22-fold at an EE-S concentration of 100 µM. EE-S also significantly stimulated MRP3-mediated uptake of EE-G (5-fold) at an EE-S concentration of 20 µM but this stimulatory effect was decreased at higher concentration (Fig. 7C). In contrast, EE-S had no stimulatory effect on MRP1-mediated EE-G uptake (Fig 7A). Such a differential modulatory effect of EE-S on uptake of EE-G by MRP1-3 suggests that the modulator binding sites vary greatly between MRP1-3 in terms of their affinity to various substrates.

To further investigate the mechanism explaining the stimulatory effect of EE-S on MRP2 and MRP3-mediated uptake of EE-G, we compared the kinetic parameters of EE-G uptake in the presence and absence of EE-S. In the presence of EE-S (20 µM), the K_m values for EE-G uptake by MRP2 and MRP3 were considerably decreased compared

with those in the absence of EE-S, while the V_{max} values were only slightly higher than those in the absence of EE-S (Fig. 3 and 8). The V_{max}/K_m, which represents the intrinsic clearance of EE-G transport, was calculated to be 110 in the presence of EE-S (20 μM) and 8.8 in the absence of EE-S for MRP2-mediated uptake, while the V_{max}/K_m ratio was 180 in the presence of EE-S (20 μM) compared to 6.3 in the absence of EE-S for MRP3-mediated uptake. Taken together, these data demonstrate that EE-S stimulates MRP2 and MRP3-mediated uptake of EE-G by increasing its apparent affinity. Therefore, EE-S could potentially enhance the excretion of EE-G *in vivo* from the organs where these transporters are expressed. Although it is important to note that EE is administered to humans at a low dose (35 μg per day), local, intracellular concentrations of these metabolites might well be in the micromolar range, as studied here. Therefore, the effects of EE-S on MRP2 and MRP3-mediated transport of EE-G might also be relevant *in vivo*. Since EE-G and EE-S are highly charged, they do not readily diffuse over the plasma membrane. We therefore could not investigate the effect of EE-S on MRP2 and MRP3-mediated EE-G transport in intact cells by (vectorial) transport studies.

Earlier studies have shown that some modulators of MRP1 and MRP2 were co-transported with substrates (Loe et al., 1998; Evers et al., 2000). The stimulation of MRP2 and MRP3-mediated uptake by EE-S raises the possibility that EE-S might be co-transported with the MRP2 and MRP3 substrates EE-G, $E_2 17\beta G$, and EA-SG. No ATP-dependent uptake of EE-S was observed in the presence of EE-G (Fig. 9), however, suggesting that EE-S was not co-transported with EE-G. We also determined that EE-S was not co-transported with $E_2 17\beta G$ in MRP3 containing vesicles (data not shown). We can not rule out that some ATP-dependent co-transport of EE-S with EE-G occurs, which we might have missed in our studies due to the relatively high non-specific binding of EE-S to membrane filters. However, even if this would be the case, the transport of

EE-S in the presence of EE-G was very weak compared to its strong stimulatory effect on EE-G transport. Taking together, the above studies suggest that EE-S has an allosteric effect on MRP2 and MRP3 resulting in an increased transport rate of EE-G, and that EE-S is not transported itself at an appreciable rate.

EE-S showed strong stimulatory effects on both MRP2 and MRP3-mediated EE-G and $E_2 17\beta G$ uptake. Hirohashi et al. (1999) and Akita et al. (2001) found that the sulfate conjugates of E3040 and 4-methyl umbelliferone also stimulated both rat and human MRP3-mediated $E_2 17\beta G$ uptake. Our studies demonstrated that the stimulatory effect of EE-S on the uptake of EE-G and $E_2 17\beta G$ varied between MRP2 and MRP3. EE-S showed a stimulatory effect on MRP2-mediated uptake of EE-G and $E_2 17\beta G$ at all concentrations tested (Fig. 6B, 7B), whereas EE-S only showed a strong stimulatory effects on MRP3-mediated EE-G and $E_2 17\beta G$ uptake at lower concentration (< 20µM). This stimulation by EE-S decreased at higher concentrations (Fig. 6C, 7C). This suggests that MRP2 and MRP3 might have a different binding affinity for EE-S. The finding that EE-S showed a much stronger stimulatory effect on MRP2-mediated EE-G and $E_2 17\beta G$ uptake than on EA-SG uptake suggests that sulfate conjugates may stimulate the transport of glucuronides stronger than of glutathione conjugates. Further experiments are needed, however, to verify this possibility.

Recently, various co-transport and allosteric activation models have been proposed to explain the complex interactions between MRP1-3 and substrates. Evers et al. (2000) proposed a working model to explain the co-transport of MRP2 substrates, including sulfinpyrazone or vinblastine, with GSH. However, this model does not explain the lack of co-transport of EE-S with EE-G and other MRP2 and MRP3 substrates, including $E_2 17\beta G$. Similarly, several recent studies also indicated that some MRP1-3 modulators are not detectably co-transported with MRP substrates whereas they had a

strong stimulatory effect (Leslie et al., 2001, 2003; Qian et al., 2001; Zelcer et al., 2003b).

While this work was in progress, Zelcer et al (2003b) proposed a transport model for MRP2, in which MRP2 has two similar but non-identical ligand binding sites: one site from which substrate is transported (S site) and another site able to modulate the transport (M site). Binding of a modulator to the M site would induce a structural change that results in a better fit of the substrate at the S site. According to this model, EE-S might bind only to the M site of MRP2, leading to a conformational change in the S site and therewith causing a strong stimulation of MRP2-mediated transport of EE-G. This model may not explain all our experiments, however. For instance, EE-S stimulated MRP2-mediated uptake of EA-SG at relatively low EE-S concentrations (2-20 μM), but uptake was inhibited at higher concentrations of EE-S (Fig. 6). Based on the "bell-shaped" stimulation curve, we speculate that EE-S could bind to both the M and S site. Therefore, at lower concentrations, it predominantly binds to the M site and stimulates EA-SG uptake; at higher concentrations, it competes with EA-SG for the S site resulting in the inhibition of EA-SG transport. At present, we have no evidence indicating that the competition between EE-S and EA-SG for the S site could result in transport of EE-S. On the other hand, EE-G might have a much higher binding affinity for the S site than EA-SG. Therefore, also at high concentrations, EE-S can not compete with EE-G for binding to the S site. Like for MRP2, our results also show that EE-S allosterically stimulates MRP3-mediated transport of EE-G and E$_2$17βG, but was not co-transported with these compounds. We infer that MRP3 might contain similar multiple ligand-binding sites as postulated for MRP2. Taken together, these observations suggest that binding of some compounds to MRP2 and MRP3 has an allosteric effect resulting in a conformational change in the protein and increasing its affinity for substrates.

In our studies, uptake of EE-G by MRP2 and MRP3 followed simple Michaelis-Menten kinetics. Also upon close inspection of the data from multiple experiments, we were not able to observe a sigmoidal uptake rate versus concentration curve as found by Zelcer et al., (2003b) and Bodo et al., (2003) for MRP2-mediated uptake of $E_217\beta G$. To explain this apparent discrepancy, we hypothesize that EE-G only binds to the S site and has no detectable affinity for the M site.

Our data suggest that the transporters responsible *in vivo* for the export of EE-G from enterocytes and hepatocytes could be MRP2 and MRP3. As several organic anions, which show stimulation of MRP-mediated uptake, are physiologically or pharmacologically active, it has been speculated that these might cause clinically relevant drug-drug interactions (Bakos et al., 2000; Huisman et al., 2002). It is important to note, however, that all these studies were performed *in vitro* with either inside-out membrane vesicles or polarized cell lines. In future studies, it therefore will be important to address whether these effects can be mimicked in animals or perfused organ systems.

The complex interaction between MRP transporters and organic anions suggests that caution needs to be taken in using inhibition studies as a screening tool to predict whether compounds could cause inhibition of MRP-mediated transport under physiological conditions. Probably such experiments should be performed with physiologically relevant substrates, like *e.g.* bilirubin glucuronide for MRP2.

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Legends for figures

Fig. 1. Western blot detection of MRP1, MRP2 and MRP3 in membrane vesicles prepared from baculovirus infected Sf9 cells.

Isolated membrane vesicles (0.5 and 2.5 µg) from baculovirus infected Sf9 cells were separated in a 7.5% denaturing polyacrylamide gel and immuno-blotted as described in the Materials and Methods section. Lanes 1 and 2 contained control membranes from β-galactosidase expressing cells (Sf9-C). Lanes 3 and 4, 5 and 6, and 7 and 8 contained membranes isolated from cells expressing MRP1, MRP2 or MRP3 cDNA, respectively. Panel A, detection of MRP1 by the anti-MRP1 monoclonal antibody, MRPr1; Panel B, detection of MRP2 by the anti-MRP2 monoclonal antibody, M_2I-4; and Panel C, detection of MRP3 by the anti-MRP3 monoclonal antibody, $MRPIII_{21}$.

Fig. 2. Time course of ATP-dependent uptake of [^{3}H] EE-G (0.2 µM) by membrane vesicles containing MRP1, MRP2 and MRP3.

Membrane vesicles (10 µg of protein) prepared from Sf9 cells expressing β-galactosidase (circles; Panels A-C), MRP1 (squares; Panel A), MRP2 (squares; Panel B) or MRP3 (squares; Panel C) were incubated at 37°C in transport buffer containing [^{3}H] EE-G (0.2 µM) in the presence of 5 mM ATP (closed symbols) or 5 mM AMP (open symbols) and an ATP-regenerating system. Values shown are means ± SE of experiments performed in triplicate.

Fig. 3. Kinetic analysis of MRP2- and MRP3-mediated [^{3}H] EE-G uptake

Kinetic parameters of [^{3}H] EE-G uptake by Sf9 cells membrane vesicles (10 µg of protein) containing MRP2 (A) or MRP3 (B) were measured at EE-G concentrations ranging from 0.2-200 µM for 5 min at 37°C with 5 mM ATP or AMP and an ATP-regenerating system. The ATP-dependent uptake was calculated by subtracting the uptake in the presence of 5 mM AMP from that in the presence of 5 mM ATP. Kinetic

parameters were obtained by fitting the uptake data to the Michaelis-Menton equation (equation 1) by *kaleida graph*. Values shown are means ± SE of experiments performed in triplicate.

Fig. 4. Time course of ATP-dependent uptake of [^{3}H] EE-S (0.2 μM) by membrane vesicles containing MRP1, MRP2 and MRP3.

Membrane vesicles (10 μg of protein) prepared from Sf9 cells expressing β-galactosidase (circles), MRP1 (squares) (Panel A), MRP2 (squares) (Panel B) (squares) or MRP3 (Panel C) (squares) were incubated at 37°C in transport buffer containing [^{3}H] EE-S (0.2 μM) in the presence of 5 mM ATP (closed symbols) or 5 mM AMP (open symbols) and an ATP-regenerating system. Values shown are means ± SE of experiments performed in triplicate.

Fig. 5. Effects of EE-G on ATP-dependent uptake of [^{14}C]EA-SG and [^{3}H] E$_2$17βG in MRP2 and MRP3 containing vesicles

Membrane vesicles (10 μg of protein) prepared from Sf9 cells expressing MRP2 (Panel A) or MRP3 (Panel B) were incubated at 37°C with 5 mM ATP or AMP and ATP-regenerating system in transport buffer containing [^{14}C] EA-SG (2 μM) (Panel A) or [^{3}H] E$_2$17βG (0.4 μM) (Panel B) and varying concentrations of EE-G for 5 min. ATP-dependent uptake was obtained by subtracting the uptake in the presence of AMP from that in the presence of ATP. Data were fitted to equation 2. Values shown are means ± SE of experiments performed in triplicate.

Fig. 6. Stimulation of MRP2-mediated uptake of EA-SG and E$_2$17βG and MRP3-mediated uptake of E$_2$17βG by EE-S

Membrane vesicles (10 μg of protein) from Sf9 cells expressing MRP2 (A, B) or MRP3 (C) were incubated at 37°C with 2 μM [^{14}C] EA-SG (A) or 0.4 μM [^{3}H] E$_2$17βG (B, C) for 5

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min in the absence and presence of different concentrations of EE-S. The ATP-dependent initial uptake rate by MRP2 or MRP3 was obtained by subtracting the transport in the presence of 5 mM AMP from the transport in the presence of 5 mM ATP. Data are expressed as fold-control uptake in the absence of EE-S. Values shown are means ± SE of experiments performed in triplicate.

Fig. 7. Stimulation of MRP1, MRP2 and MRP3-mediated uptake of EE-G by EE-S

Membrane vesicles (10 µg of protein) from Sf9 cells expressing MRP1 (Panel A), MRP2 (Panel B) or MRP3 (Panel C) were incubated at 37°C with 0.2 µM [^{3}H] EE-G for 5 min in the presence of 5 mM ATP or AMP and an ATP-regenerating system in transport buffer. Before the incubation of the vesicles, EE-S was added at concentrations of 0, 2, 5, 20, 50, and 100 µM, respectively, and preincubated with the ligand, ATP or AMP and the ATP-regenerating system at 37 ^{o}C for 3 min. The ATP-dependent initial uptake rate of EE-G was obtained by subtracting the transport in the presence of 5 mM AMP from the transport in the presence of 5 mM ATP. Data are expressed as fold-control uptake in the absence of EE-S. Values shown are means ± SE of experiments performed in triplicate.

Fig. 8. Kinetic analysis of MRP2- and MRP3-mediated [^{3}H] EE-G uptake in the presence of 20 µM EE-S

Kinetic parameters of [^{3}H] EE-G uptake by Sf9 cells membrane vesicles (10 µg of protein) containing MRP2 (A) or MRP3 (B) were measured at EE-G concentrations ranging from 0.2-200 µM for 5 min at 37°C with 5 mM ATP or AMP and an ATP-regenerating system in the presence of 20 µM EE-S. Experimental details as in Fig. 3.

Fig. 9. Effect of EE-G on MRP2 and MRP3 mediated uptake of EE-S

Membrane vesicles (10 µg of protein) containing MRP2 (Panel A) or MRP3 (Panel B) were incubated at 37 ^{o}C with 20 µM [^{3}H] EE-S for 5 min in the presence of 5 mM ATP (closed symbols) or AMP (open symbols) and ATP-regenerating system in transport

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buffer. Before the incubation of the vesicles, EE-G was added at a final concentration of

0, 0.5, 5, 20, and 100 µM, respectively, and preincubated with the ligand, ATP or AMP

and the ATP-regenerating system at 37 ^{o}C for 3 min. Values shown are means ± SE of

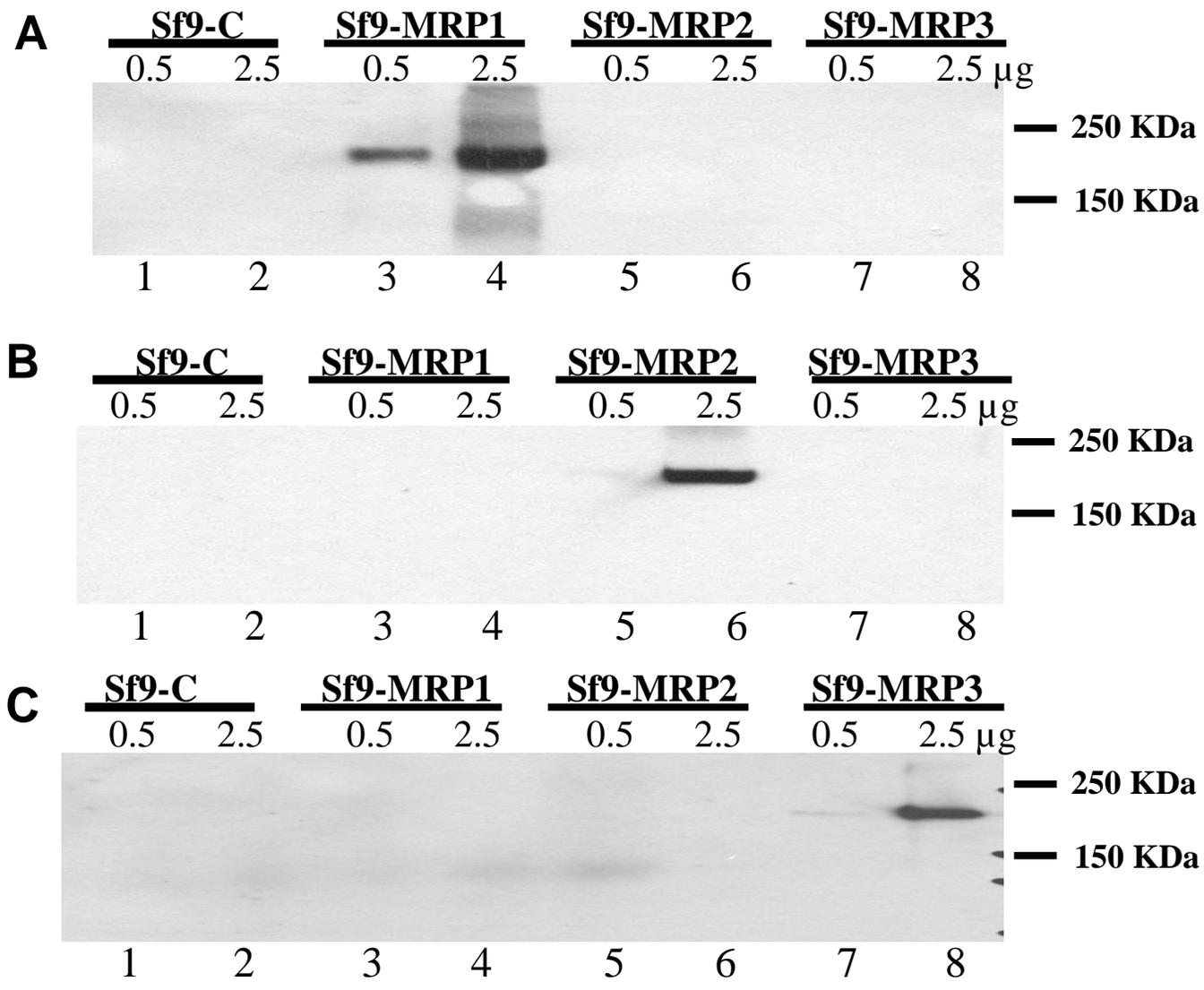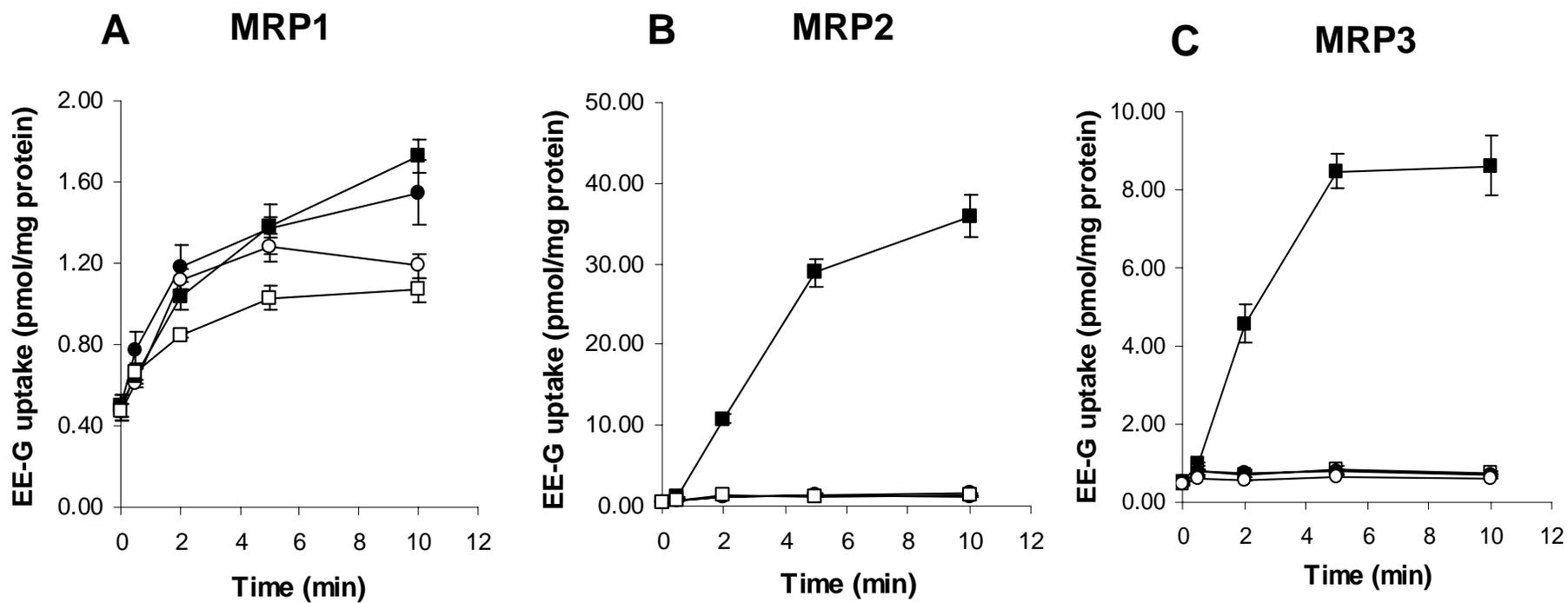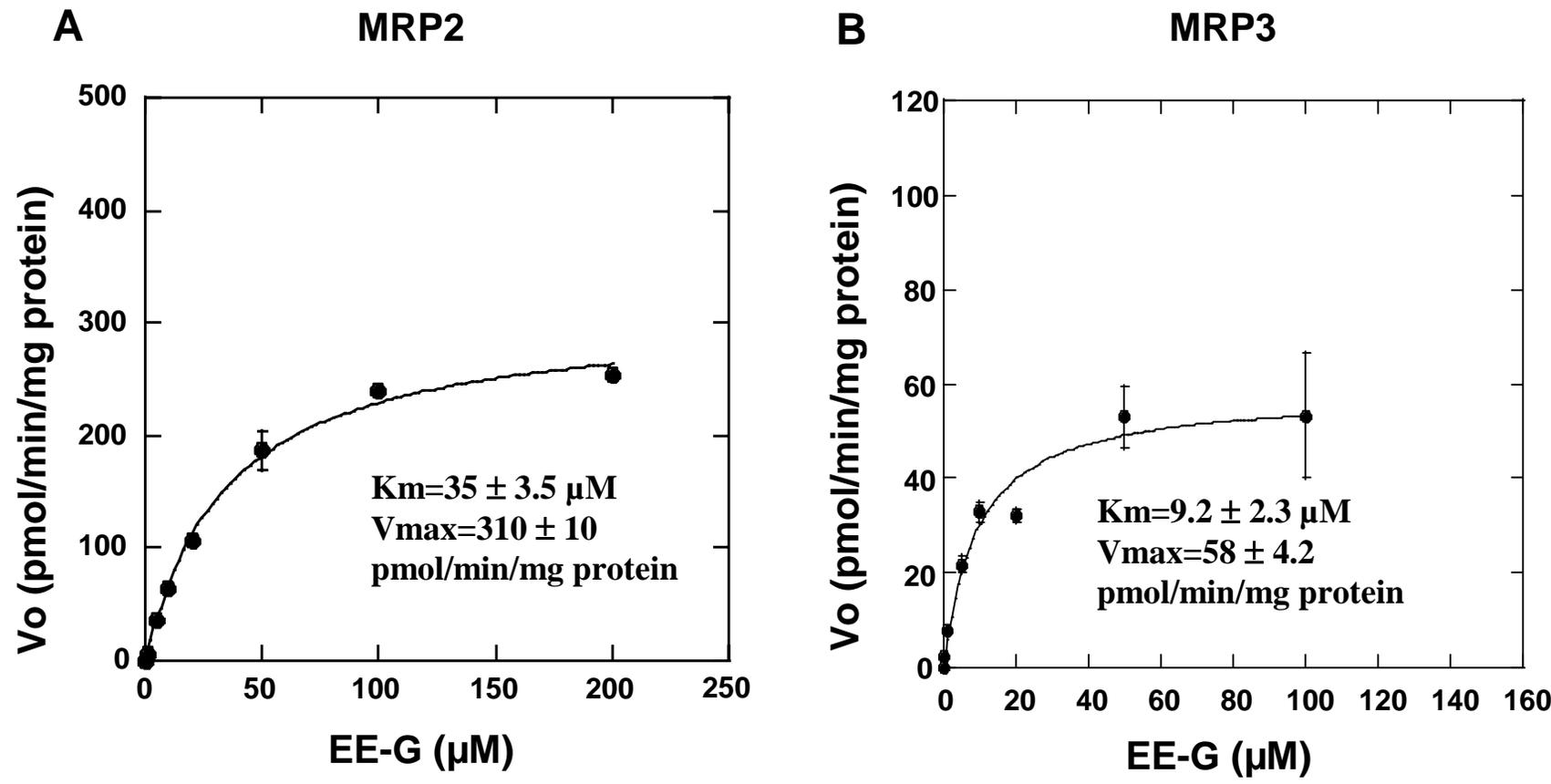experiments performed in triplicate.

Figure 1 Chu et al.

A   MRP1

B   MRP2

C   MRP3

Figure 2 Chu et al.

A   **MRP2**

Km=35 ± 3.5 µM
Vmax=310 ± 10
pmol/min/mg protein

EE-G (µM)

B   **MRP3**

Km=9.2 ± 2.3 µM
Vmax=58 ± 4.2
pmol/min/mg protein

EE-G (µM)
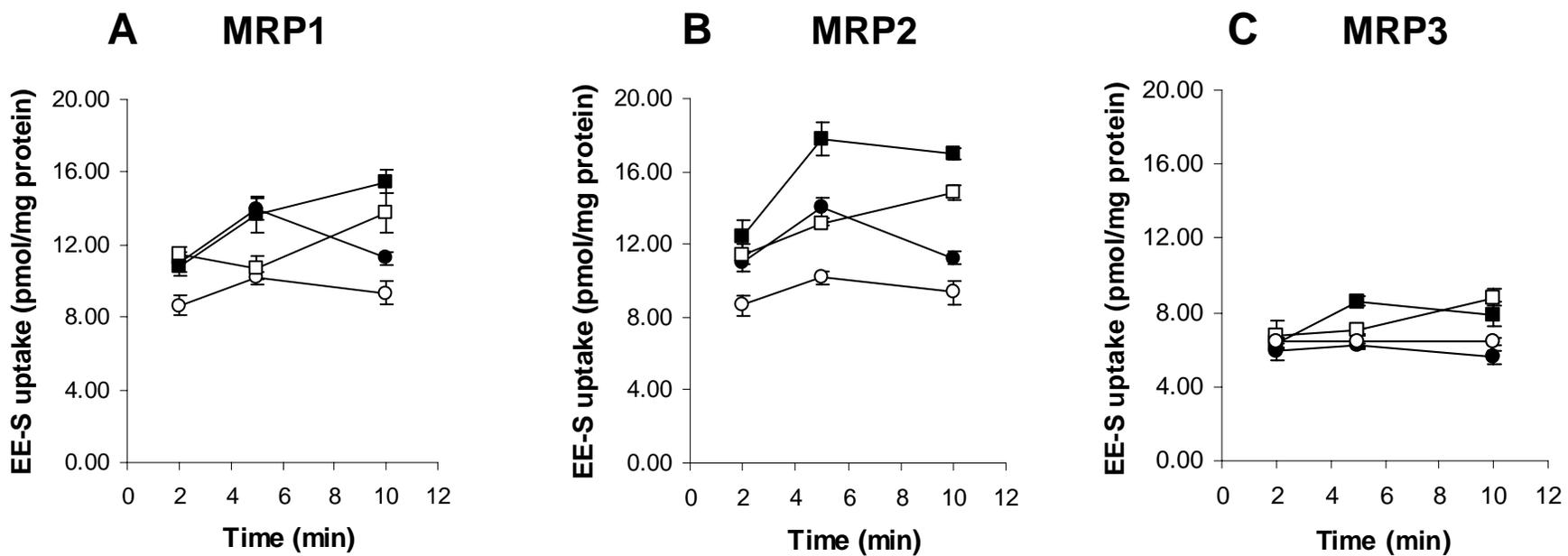
Figure 3 Chu et al.

Figure 4 Chu et al.

Figure 5 Chu et al.

A

MRP2

B

MRP2

C

MRP3

Figure 6 Chu et al.

A

MRP1

B

MRP2

C

MRP3

Figure 7 Chu et al.

A   **MRP2+ 20µM EE-S**

Km=4.1 ± 0.7 µM
Vmax=435 ± 16
pmol/min/mg protein

B   **MRP3+ 20µM EE-S**

Km=0.5 ± 0.2 µM
Vmax=88± 5.6
pmol/min/mg protein

Figure 8 Chu et al.

A

MRP2

B

MRP3

Figure 9 Chu et al.