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# Interactions of the Human Multidrug Resistance Proteins MRP1 and MRP2 with Organic Anions

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## ABSTRACT

The human multidrug resistance protein MRP1 and its homolog, MRP2, are both suggested as being involved in cancer drug resistance and the transport of organic anions. We expressed MRP1 and MRP2 in *Spodoptera frugiperda* ovarian cells and compared their ATP-dependent transport properties and vanadate-sensitive ATPase activities in isolated membrane vesicles. Both MRP1 and MRP2 actively transported leukotriene $C_4$ and *N*-ethylmaleimide glutathione (NEM-GS), although the relative affinity of MRP2 for these substrates was found to be significantly lower than that of MRP1. Methotrexate was actively transported by both proteins, although more efficiently by MRP2. ATP-dependent NEM-GS transport by MRP1 and MRP2 was variably modulated by organic anions. Probenecid and furosemide inhibited, whereas under certain conditions sulfinpyrazone, penicillin G, and indomethacin greatly stimulated, MRP2-mediated NEM-GS uptake. Vanadate-sensitive ATPase activity in isolated membranes containing MRP1 or MRP2 was significantly stimulated by NEM-GS and reduced GS, although these compounds acted only at higher concentrations in MRP2. ATP hydrolysis by MRP2 was also effectively stimulated by methotrexate. Probenecid, sulfinpyrazone, indomethacin, furosemide, and penicillin G all significantly increased MRP2-ATPase activity, whereas these compounds acted more as ATPase inhibitors on MRP1. These results indicate that MRP1 is a more efficient transporter of glutathione conjugates and free glutathione than MRP2, whereas several anions are preferred substrates for MRP2. Our data suggest that MRP2 may be responsible for the active secretion of pharmacologically relevant organic anions, such as diuretics and antibiotics, and indicate different modulation possibilities for MRP1 or MRP2 in drug-resistant tumor cells.

The human multidrug resistance proteins 1 and 2 [MRP1 and MRP2 (multispecific organic anion transporter), respectively] are homologous members of a subfamily of the ATP-binding cassette transporters, and both may cause multiple drug resistance in malignant tumor cells (Cole et al., 1992; Zaman et al., 1994; Cui et al., 1999). By now, at least six members of this subfamily have been identified, and they seem to play an important role in various secretory and other transport functions, predominantly in epithelial cells (Borst et al., 1997; Kool et al., 1997; Cui et al., 1999). Both MRP1 and MRP2 were shown to perform an ATP-dependent, primary active transport of the glutathione (GS) conjugate leukotriene $C_4$ ($LTC_4$) and of various GS, sulfate, and glucuronide conjugates (Jedlitschky et al., 1994, 1996, 1997; Müller et al., 1994). It is most likely that MRP1 and MRP2 can also transport hydrophobic drugs (Cole et al., 1994; Holló et al., 1996; Evers et al., 1998), although cellular GS seems to be an important modulator in these transport functions (see Zaman et al., 1995; Loe et al., 1996, 1998).

The physiological role of these highly promiscuous transporters may cover a wide range, varying from the transport of excretory compounds and the elimination of xenobiotics, to the mediation of an inflammatory response. The widely expressed MRP1 has a key function in, for example, $LTC_4$-dependent tissue reactions, as well as in controlling transport across the blood-brain barrier (Wijnholds et al., 1997; Rao et al., 1999), and in polarized cells, this protein is sorted to the basolateral membranes (see Borst et al., 1997; Deeley and Cole, 1997). MRP2 is predominantly expressed in the canalicular (apical) membranes of hepatocytes and the epithelial cells of kidney proximal tubules (Schaub et al., 1997; Evers et al., 1998). This protein was shown to be the most important exporter of conjugated bile salts in the liver

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**ABBREVIATIONS:** MRP1, human multidrug resistance protein 1; MRP2, human multidrug resistance protein 2; $LTC_4$, leukotriene $C_4$; GS, glutathione; GSH, reduced glutathione; MDR1, human multidrug resistance protein (P-glycoprotein); NEM, *N*-ethylmaleimide; *Sf*9, *Spodoptera frugiperda* 9.

(Büchler et al., 1996; Paulusma et al., 1996; Jedlitschky et al., 1997). MRP1 definitely, and MRP2 probably, plays an important role in the chemotherapy resistance of several types of cancer cells (see Deeley and Cole, 1997; Kool et al., 1997). Therefore, the determination of the substrate interactions with these two transporters is of major importance for the understanding of the cellular pharmacology and toxicology of a wide variety of compounds, as well as for the proper planning and adjustment of cancer chemotherapy.

In the present study, we expressed MRP1 and MRP2 in baculovirus-infected *Spodoptera frugiperda* ovarian (*Sf*9) insect cells and measured the ATP-dependent, vanadate-sensitive transport of two established MRP substrates, $LTC_4$ and *N*-ethylmaleimide (NEM)-GS, as well as of the organic anion anticancer agent methotrexate, in isolated membrane vesicles. In the insect cell membrane preparations, we also examined the effect of various anionic compounds on the specific, vanadate-inhibitable ATPase activity of MRP1 and MRP2. Both human MRP1 (Bakos et al., 1996, 1998; Gao et al., 1996) and MRP2 from rabbit (van Aubel et al., 1998) have been successfully expressed in baculovirus-infected insect cells, and although underglycosylated, their basic structural and transport characteristics were found to be identical to those seen in mammalian cells. Because high-level heterologous protein expression makes the involvement of any complementary or closely related endogenous transporter unlikely, direct transport measurements in isolated insect cell membrane vesicles can be most helpful in establishing the relative affinities and transport rates for various substrates of these human transporters. Drug-stimulated ATPase in insect cell membranes was first applied to characterize the enzymatic function of human multidrug resistance protein (P-glycoprotein) (MDR1; Sarkadi et al., 1992), and since then, it has proved to be a valuable tool for the simple and efficient screening of substrate-transporter interactions in numerous studies (see Scarborough, 1995; Germann, 1998).

Our experiments were prompted by the data obtained with MRP1- and MRP2-expressing polarized mammalian cells (R.E., M. de Haas, R. Sparidans, J. Beijnen, P. R. Wielinga, J. Lankelma, and P.B., unpublished data), which indicated major differences between the transport properties of MRP1 and MRP2. In accordance with those data, the results in the present report suggest that several non-GS-conjugate anionic pharmacons are efficiently transported by MRP2, whereas these compounds act mostly as inhibitors on MRP1. Several organic anions (e.g., probenecid, sulfinpyrazone, indomethacin, furosemide, and penicillin G) are actively secreted over the apical membrane of the proximal tubules of the kidney. Because MRP2 is predominantly expressed in this region of the kidney (Schaub et al., 1997), our experiments suggest that MRP2 may be a key player in this secretion. This information may facilitate the proper planning of pharmacological interventions in diseases related to the altered metabolism, distribution, and transport of organic anions, as well as the elimination of transporter-specific multidrug resistance in cancer cells.

## Experimental Procedures

**Materials.** [$^3$H]$LTC_4$ (135 Ci/mmol) and [$^3$H]methotrexate (15 Ci/mmol) were obtained from DuPont-New England Nuclear (Boston, MA) and Moravek Biochemicals (Brea, CA), respectively. [$^3$H]NEM-GS was prepared from [$^3$H]NEM (60 Ci/mmol; DuPont-New England Nuclear) by mixing the isotope in 10 mM Tris-HCl (pH 7.0) with freshly dissolved reduced GS (GSH) in a 1:1.1 molar ratio.

**Expression of MRP1 and MRP2 in Insect Cells.** Recombinant baculoviruses containing the MRP1 cDNA were prepared as described by Bakos et al. (1998) by using the BaculoGold Transfection Kit (PharMingen, San Diego, CA). MRP2 baculoviruses were prepared similarly: MRP2 cDNA (Paulusma et al., 1996) from modified pGEM-MRP2 (to remove an out-of-frame upstream start codon, the sequence of the 5′ UTR was changed to CTTTAAAAATACAAA using polymerase chain reaction) was removed by digestion with *Hin*dIII and *Nco*I and subcloned into the pAcUW21 plasmid (InVitrogen, San Diego, CA). *Sf*9 cells were cultured and infected with a baculovirus as described in Müller et al. (1996).

**Membrane Preparation and Immunoblotting.** Virus-infected *Sf*9 cells were harvested, their membranes were isolated and stored, and the membrane protein concentrations were determined as described in Sarkadi et al. (1992). Immunoblotting was performed after dissolving and sonicating the isolated membranes in a disaggregation buffer. MRP1 was detected with the monoclonal antibody MRP1 M6 (Flens et al., 1996), and MRP2 was detected with the monoclonal antibody $M_2$-III-6. Protein-antibody interaction was determined using the enhanced chemiluminescence technique as described previously (Bakos et al., 1998). For the detection of MRP1 and MRP2 in mammalian cells, we used S1-MRP1-transfected and MDCKII-MRP2-transfected cells (Zaman et al., 1994; Evers et al., 1998).

**Transport Measurements.** [$^3$H]NEM-GS, [$^3$H]$LTC_4$, and [$^3$H]methotrexate transport measurements in isolated *Sf*9 cell membrane vesicles were performed as described earlier by Bakos et al. (1998). In brief, vesicles were incubated in the presence of 4 mM ATP or AMP in a buffer containing 10 mM $MgCl_2$, 40 mM 3-(*N*-morpholino)propanesulfonic acid-Tris (pH 7.0), and 50 mM KCl at 23°C ($LTC_4$) or at 37°C (NEM-GS, methotrexate). Aliquots of this suspension were added to excess cold transport buffer and then rapidly filtered through 0.25-$\mu$m-pore nitrocellulose membranes. The filters were washed extensively, and radioactivity associated with the filters was measured by liquid scintillation counting. ATP-dependent transport was calculated by subtracting the values obtained in the presence of AMP from those in the presence of ATP. The figures represent mean values from three independent experiments.

**Membrane ATPase Measurements.** ATPase activity was measured basically as described by Sarkadi et al. (1992) by determining the liberation of inorganic phosphate from ATP with a colorimetric reaction. The incubation media contained 10 mM $MgCl_2$, 40 mM 3-(*N*-morpholino)propanesulfonic acid-Tris (pH 7.0), 50 mM KCl, 5 mM dithiothreitol, 0.1 mM EGTA, 4 mM Na-azide, 1 mM ouabain, and 4 mM ATP. The final membrane protein concentration was 200 $\mu$g/ml. The incubation time was 60 min at 37°C. Special care was taken to avoid any possible changes in the pH of the assay medium at higher organic anion concentrations. The figures represent mean values from three independent experiments.

## Results

**Expression of MRP1 and MRP2 in Insect Cells.** Figure 1 demonstrates that both MRP1 and MRP2 were successfully expressed in *Sf*9 cells. According to our estimation from several similar immunoblotting studies, the isolated *Sf*9 cell membranes contained about 20 times higher levels of these proteins than the corresponding, highly drug-resistant S1-MRP1 or MDCKII-MRP2 cell membranes. In *Sf*9 cells, both proteins were produced in an underglycosylated form, which has been demonstrated to not affect their transport functions (Gao et al., 1996; Bakos et al., 1998; van Aubel et al., 1998). The exact comparison of the expression levels of the proteins in *Sf*9 cells could not be performed because we had no monoclonal antibody recognizing equivalent epitopes in MRP1 and

MRP2. Based on gel staining, chimera protein expression studies,[2] and the following transport and ATPase data, the *Sf*9 cell membrane expression levels were estimated to be roughly similar for MRP1, MRP2, and those measured formerly for MDR1.

**Transport Measurements in Membrane Vesicles.** To compare the transport characteristics of MRP1 and MRP2, we studied the uptake of the radiolabeled GS conjugates $LTC_4$ and NEM-GS, as well as the anticancer drug methotrexate, in isolated *Sf*9 cell membrane vesicles. As demonstrated previously, the relative amount of *Sf*9 membrane vesicles and their transport competence were not affected by the expression of various foreign membrane proteins (Bakos et al., 1998). Examination of the transport of each labeled compound in control, β-galactosidase-expressing *Sf*9 cell membranes showed that the ATP-dependent tracer uptake was negligible. Also, the addition of 1 M sucrose to the assay media, causing shrinkage of the vesicles, eliminated ATP-dependent transport in all experiments. For the characterization of the function of MRP1 or MRP2 (i.e., for calculating the transport rates), in each case the linear phase of the tracer uptake (20 s for $LTC_4$ and 4 min for methotrexate and NEM-GS; see Bakos et al., 1998) was used. ATP-dependent tracer uptake was calculated by subtracting the values measured in the presence of AMP.

We found that both MRP1 and MRP2 efficiently transported $LTC_4$ and NEM-GS, but the concentrations of these compounds at which half-maximum uptake rates were reached ($K_{1/2}$) were significantly higher for MRP2 than for MRP1. The $K_{1/2}$ for $LTC_4$ uptake of MRP1 was 130 to 150 nM (see also Bakos et al., 1998), whereas that of MRP2 was 1 to 1.4 μM (not shown). The transport data for NEM-GS and methotrexate are shown in detail in Fig. 2, A and B. The $K_{1/2}$ value for ATP-dependent NEM-GS uptake in MRP1-containing vesicles was about 200 μM, whereas this value was about 2.5 mM in MRP2-containing membranes. In contrast to $LTC_4$ and NEM-GS, methotrexate was significantly more efficiently transported by MRP2, with an approximate $K_{1/2}$ of 2.5 to 3 mM. For MRP1, because of the low transport rates, the $K_{1/2}$ value could not be exactly determined. Still, even at high methotrexate concentrations, the transport rate by MRP1 did not approach the level of NEM-GS uptake by the same transporter. When examining the effects of free GSH on methotrexate uptake, we found no stimulation of this transport for either MRP1 or MRP2, whereas GSH at more than 10 mM caused a slight inhibition of vesicular methotrexate uptake (not shown).

Because the active secretion of organic anions in the kidney proximal tubules may involve MRP2 (see *Discussion*), we also studied the effects of some other known secreted organic anions and secretion inhibitors on the NEM-GS transport by MRP1 and MRP2. Furosemide and penicillin G are known substrates of this secretory pathway, which is competitively inhibited by probenecid and sulfinpyrazone and probably noncompetitively by benzbromarone.

Figure 3 shows the effects of various organic anions on the relative rate of NEM-GS uptake by MRP1 and MRP2. In the

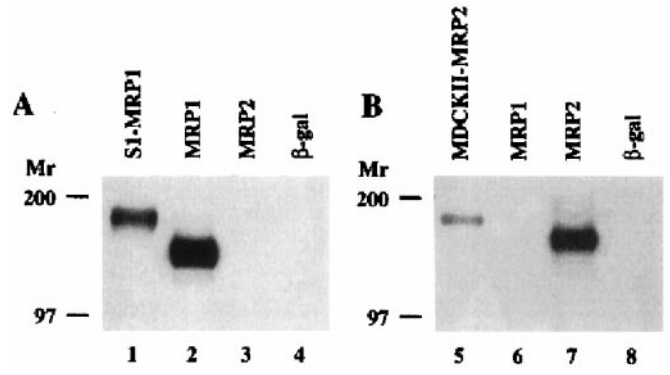


**Fig. 1.** Immunoblot detection of MRP1 and MRP2 by monoclonal antibodies. A, detection by the anti-MRP1 monoclonal antibody, M6. B, developed by the anti-MRP2 monoclonal antibody $M_2$-III-6. Isolated membranes were subjected to electrophoresis and immunoblotting as described in *Experimental Procedures*. The samples were obtained from S1 MRP1 cells (lane 1, 10 μg protein), MDCKII-MRP2 cells (lane 5, 10 μg protein), and isolated membranes of *Sf*9 cells expressing either MRP1 (lanes 2 and 6, 1 μg protein), MRP2 (lanes 3 and 7, 1 μg protein), or β-galactosidase (lanes 4 and 8, 2 μg protein).

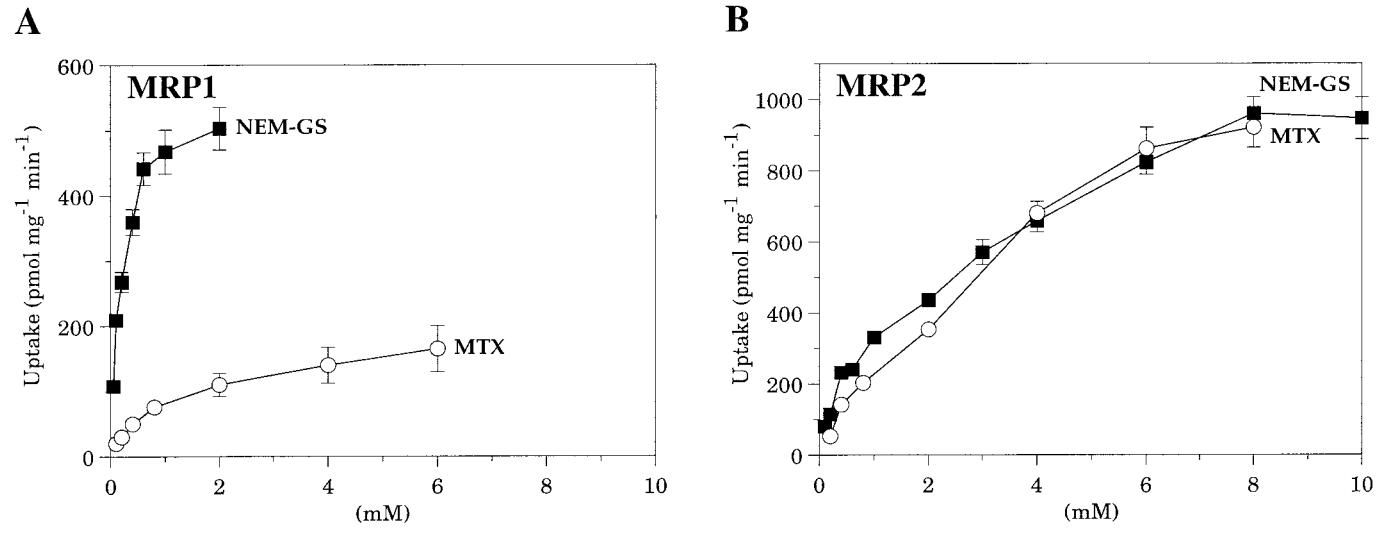


**Fig. 2.** ATP-dependent, labeled NEM-GS and methotrexate (MTX) uptake in *Sf*9 cell membrane vesicles, expressing MRP1 (A) or MRP2 (B). Membrane preparations were incubated with different concentrations of labeled NEM-GS (■) or methotrexate (MTX, ○) for 4 min at 37°C. ATP-dependent uptake was calculated by subtracting the values obtained in the presence of 4 mM AMP from those in the presence of 4 mM ATP (see *Experimental Procedures*).

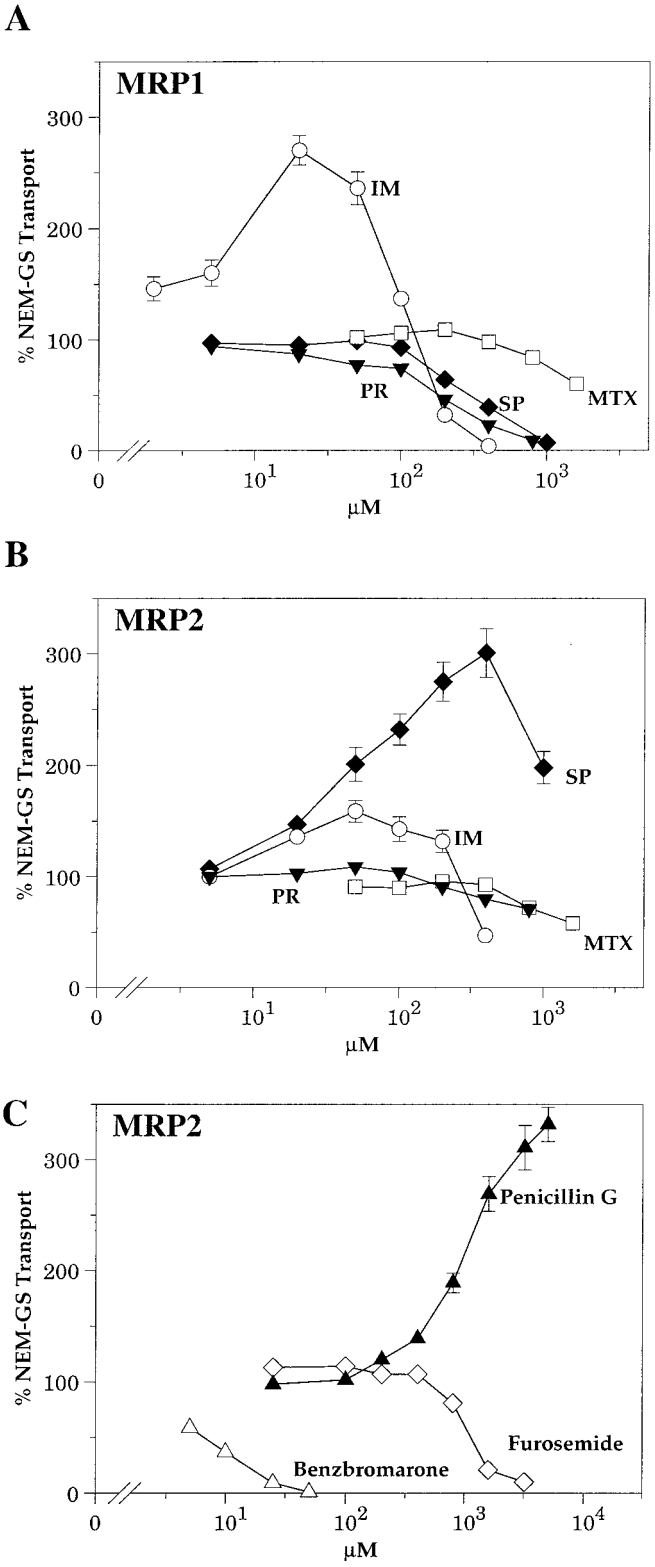


**Fig. 3.** Modulation of ATP-dependent, labeled NEM-GS uptake in *Sf*9 cell membrane vesicles, expressing MRP1 (A) or MRP2 (B and C), by organic anions. *Sf*9 cell membrane vesicles were incubated with 4 $\mu$M NEM-GS at 37°C for 4 min. ATP-dependent tracer uptake was measured by rapid filtration, and the respective transport rates were expressed as percent of the tracer uptake measured in the absence of additional compounds (percent of control; see *Experimental Procedures*). A and B, effects of probenecid (PR, (▼), sulfinpyrazone (SP, ♦), methotrexate (MTX, □), and indomethacin (IM, ○) are shown. C, effects of penicillin G (▲), benzbromarone (△), and furosemide (◇).

experiments presented in Fig. 3, tracer uptake was measured at relatively low NEM-GS concentrations (4 $\mu$M). In the case of MRP1 (Fig. 3A), we found that sulfinpyrazone and probenecid efficiently inhibited ATP-dependent active vesicular NEM-GS uptake. However, low concentrations of indomethacin produced a significant stimulation of this GS-conjugate uptake, and only indomethacin concentrations of more than 100 $\mu$M were inhibitory. In the case of MRP2 (Fig. 3, B and C), probenecid inhibited NEM-GS uptake, whereas low concentrations of sulfinpyrazone and indomethacin strongly stimulated the transport of this GS conjugate. Higher sulfinpyrazone and indomethacin concentrations were inhibitory again. The addition of methotrexate caused a slight inhibition of the NEM-GS uptake in both MRP1 and MRP2. As shown in Fig. 3C for MRP2, several other organic anions also modulated NEM-GS uptake: penicillin G caused a major stimulation of this transport, whereas benzbromarone or furosemide was inhibitory. In the case of MRP1, only the inhibition of NEM-GS transport was observed with these compounds (not shown). In similar tracer transport experiments, we also examined the effect of free GSH (2–10 mM) on the rate of NEM-GS uptake and found no significant stimulation for either MRP1 or MRP2, whereas GSH concentrations of more than 5 mM were slightly inhibitory (data not shown).

**ATPase Measurements.** The vanadate-sensitive ATPase activity of the multidrug transporter MDR1 has been shown to reflect the substrate interactions of this protein: transported substrates significantly (up to 3- to 6-fold compared with the baseline level) stimulated the ATPase activity, whereas non MDR1 substrates had no effect (see Sarkadi et al., 1992; Scarborough, 1995). In one study, Chang et al. (1998) examined the vanadate-sensitive ATPase of the purified MRP1 protein and found stimulation by GS conjugates, although stimulation was weak (1.3- to 1.5-fold). In the present experiments, by using the high-level expression of human MRP1 and MRP2 in *Sf*9 cells, we examined the effects of various compounds on the ATPase activity of both proteins in a membrane environment.

As documented in Fig. 4, the vanadate-sensitive ATPase activity of both MRP1 (Fig. 4A) and MRP2 (Fig. 4B) was relatively low in the absence of drugs (4–5 nmol/mg membrane protein/min) but significantly stimulated (reaching about 13–15 nmol/mg membrane protein/min) by NEM-GS. Membranes from cells expressing $\beta$-galactosidase had a low-level basal ATPase activity (2–3 nmol/mg membrane protein/min), and no measurable stimulation was detected by NEM-GS, GSH, or any of the other agents examined. Compared with the ATPase activity of MDR1 measured in similarly prepared *Sf*9 cell membranes (see Müller et al., 1996) and assuming similar expression levels for these human proteins, the maximum level of the ATPase activity in the case of both MRP1 and MRP2 was about 5 times smaller than that found for MDR1. In unpublished experiments, we compared the levels of MRP1 and MDR1 expression in *Sf*9 cell membranes by using chimera MDR1-MRP1 proteins and a set of MRP1- and MDR1-specific antibodies. These studies indicated comparable levels of MRP1/MDR1 expression, corresponding to about 3% of the total membrane protein. Nevertheless, for both MRP1 and MRP2, ATP hydrolysis was linear for at least 60 min. Therefore, we used this time period to analyze the ATPase activity. To ensure fully reductive assay conditions, all assays were performed in the presence of 5 mM dithio-

threitol, which had no effect on the ATPase activity. These ATPase assay conditions did not allow the determination of the concentration dependence of the chemically labile $LTC_4$ molecule, but a significant stimulation of ATPase activities by both MRP1 and MRP2 was measured at 5 $\mu$M $LTC_4$ (data not shown).

As shown in Fig. 4, the maximum level of stimulation of the ATPase activity by NEM-GS was similar for MRP1 and MRP2, but the NEM-GS concentration dependence was clearly different: the estimated concentration producing half-maximum activation ($K_{ACT}$) for MRP1 was about 400 to 500 $\mu$M, whereas this value for MRP2 approached 3 to 4 mM (we could not exactly measure the maximum ATPase rate because higher NEM-GS concentrations inhibited the inorganic phosphate assay). These ATPase activation results closely corresponded to the respective $K_{1/2}$ values found for MRP1 and MRP2 in the direct NEM-GS transport measurements. Activation of the ATPase by GSH was also different for the two transporters: at 20 mM GSH, the ATPase activity of MRP1 approached the level obtained with 5 mM NEM-GS, whereas in the case of MRP2, even 20 mM GSH produced only about 30% of the NEM-GS activated ATPase activity (again, higher GSH concentrations disturbed the phosphate assay). Figure 4 also shows the effect of methotrexate on the ATPase activity of MRP1 (Fig. 4A)- and MRP2 (Fig. 4B)-containing membranes. In the case of MRP1, at methotrexate concentrations between 0.5 and 2 mM, only a slight stimulation of the ATPase activity was observed. In contrast, methotrexate concentrations of more than 200 $\mu$M significantly stimulated the ATPase activity of MRP2.

To exclude that possible differences in ATP dependence could give rise to differences in the drug-stimulated ATPase activities, we examined the MgATP concentration dependence of the ATPase activity of both MRP1 and MRP2 in the presence of maximum-stimulating NEM-GS concentrations (5 and 10 mM, respectively). The apparent $K_m$ value for MgATP was about 0.4 mM for both MRP1 and MRP2, indicating that their relative affinities for ATP were found to be similar (data not shown). Drug modulation of the ATPase activities was measured at saturating (4 mM) MgATP concentrations in all related experiments.

In the following set of experiments, we investigated the effects of the organic anions probenecid, sulfinpyrazone, and indomethacin on the ATPase activity measured in isolated *Sf*9 cell membranes containing either MRP1 (Fig. 5A) or MRP2 (Fig. 5B). Because cellular GSH may significantly affect transport by both MRPs, we also examined the changes in ATPase activity in the presence of GSH (Fig. 6). In the case of MRP1 and MRP2, we used 5 and 10 mM GSH, respectively, to obtain only a partial stimulation of the ATPase activity of both transporters.

As documented in Fig. 5A, in MRP1-containing membranes the organic anions tested produced only a weak ATPase activation in comparison to NEM-GS. A significant inhibition of the MRP1-ATPase was observed at higher organic anion concentrations. In contrast, the ATPase activity of MRP2 (Fig. 5B) was strongly stimulated by both probenecid (approximate $K_{ACT}$ = 250 $\mu$M), sulfinpyrazone ($K_{ACT}$ = 300 $\mu$M), and indomethacin ($K_{ACT}$ = 150 $\mu$M), and ATPase activation was even stronger than in the case of NEM-GS. The organic anion activation of the MRP2-ATPase followed bell-shaped curves, with maximum values obtained at about 2 mM for probenecid, 800 $\mu$M for sulfinpyrazone, and 400 $\mu$M for indomethacin.

As shown in Fig. 6A, in MRP1-containing membranes 5 mM GSH gave a substantial ATPase activity, which was significantly inhibited by probenecid, sulfinpyrazone, and higher concentrations of indomethacin. Indomethacin at low concentrations (5–100 $\mu$M) induced a stimulation of the MRP1-ATPase activity. In MRP2-containing membranes (Fig. 6B), the stimulation brought about by 10 mM GSH alone was less pronounced than that in the case of MRP1, and the presence of GSH did not change the overall stimulatory effects of probenecid, sulfinpyrazone, or indomethacin. In general, an additive effect was observed on the ATPase activity by these organic anions and GSH.

In experiments not documented in detail, we also tested the effect of probenecid, sulfinpyrazone, and indomethacin on

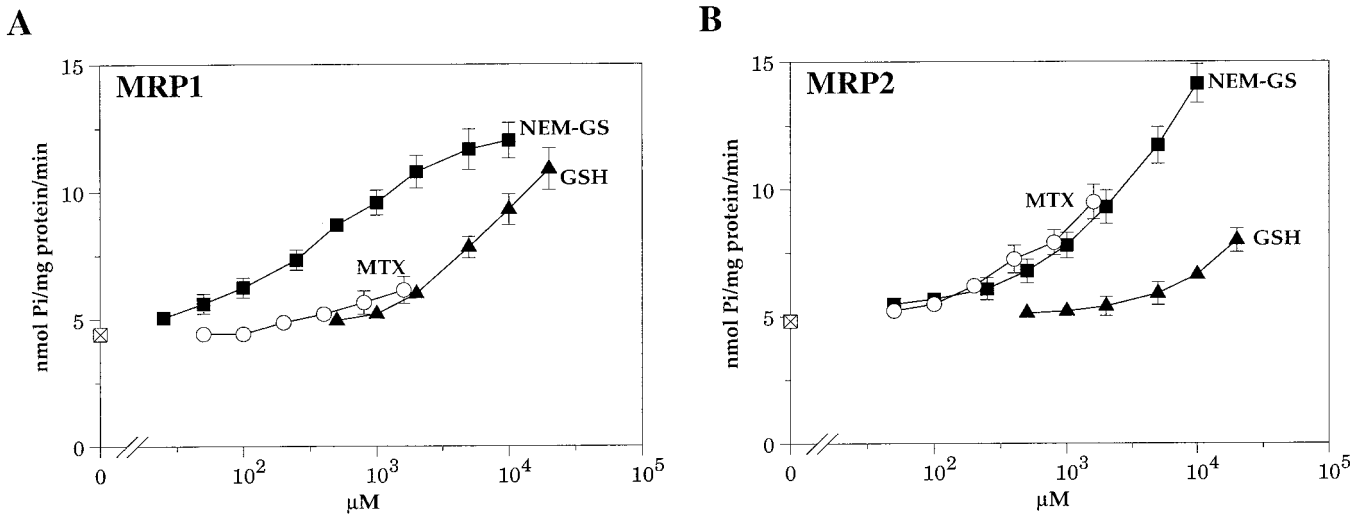


**Fig. 4.** Vanadate-sensitive ATPase activity of isolated *Sf*9 cell membranes expressing MRP1 (A) or MRP2 (B). Effects of GSH, NEM-GS, and methotrexate are shown. Membrane ATPase activity was measured for 60 min at 37°C in the presence of 4 mM ATP (control, ⊠) and various concentrations of NEM-GS (■), GSH (▲), or methotrexate (MTX, ○), as indicated on the abscissa. For details of the ATPase assay, see *Experimental Procedures*.

the membrane ATPase activities in the presence of NEM-GS concentrations (1 and 3 mM, respectively) that partially activate the ATPase of MRP1 and MRP2. Although the organic acids under these conditions inhibited the MRP1-ATPase, they significantly stimulated the MRP2-ATPase activity, clearly overriding the NEM-GS-stimulated ATPase level.

As shown earlier (Fig. 3C), several organic anions significantly modified NEM-GS transport by MRP2. Figure 7 demonstrates that ATP hydrolysis by MRP2 (and not by MRP1; data not shown) was strongly stimulated by furosemide ($K_{ACT} \sim 300$ $\mu$M, peak stimulation observed at 1 mM) and penicillin G ($K_{ACT} \sim 1.5$ mM, stimulation increasing up to 5 mM), whereas benzbromarone caused a strong inhibition of the MRP2-ATPase at concentrations above 20 $\mu$M. *Para*-aminohippuric acid (up to 3 mM) and uric acid (up to 5 mM) had no measurable effect on the ATPase activity of either MRP1 or MRP2. The presence of GSH caused an additive increase in the MRP2-ATPase in the case of all of these compounds but did not modify the overall picture of their ATPase modulation (data not shown).

## Discussion

The membrane transporter proteins of the MRP family seem to play a significant role in the cellular organic anion extrusion, especially in secretory epithelial cells. Although these transporters show a high level of structural similarity (see Tusnády et al., 1997), their physiological and pharmacological functions may be different. In polarized epithelia, MRP1 is targeted to the basolateral membranes, whereas MRP2 is targeted to the apical membranes, and their substrate interactions seem to be overlapping but nonidentical (Borst et al., 1997; Cui et al., 1999).

To study membrane protein-substrate interactions, the high-level heterologous protein expression in insect cells has numerous advantages. The *Sf*9-baculovirus expression sys-

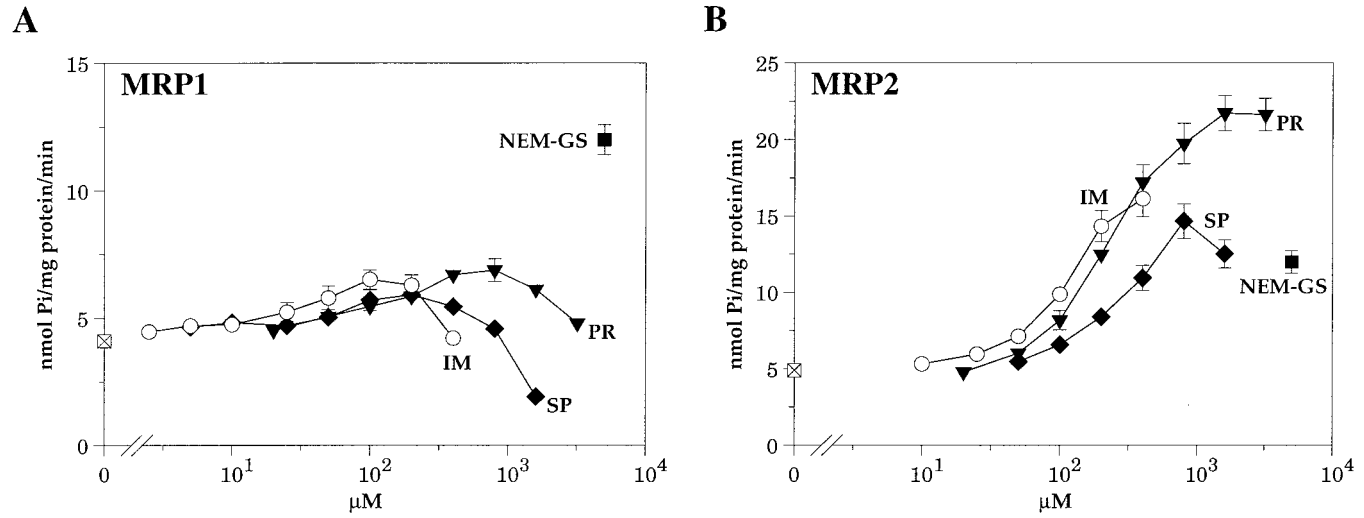


**Fig. 5.** Vanadate-sensitive ATPase activity of isolated *Sf*9 cell membranes expressing MRP1 (A) or MRP2 (B). Effects of probenecid (PR, ▼), sulfinpyrazone (SP, ♦), and indomethacin (IM, ○) are shown. Membrane ATPase activity was measured for 60 min at 37°C in the presence of 4 mM ATP (control, ⊠), as described in *Experimental Procedures*. The ATPase activity obtained in the presence of 5 mM NEM-GS (■) is also marked.

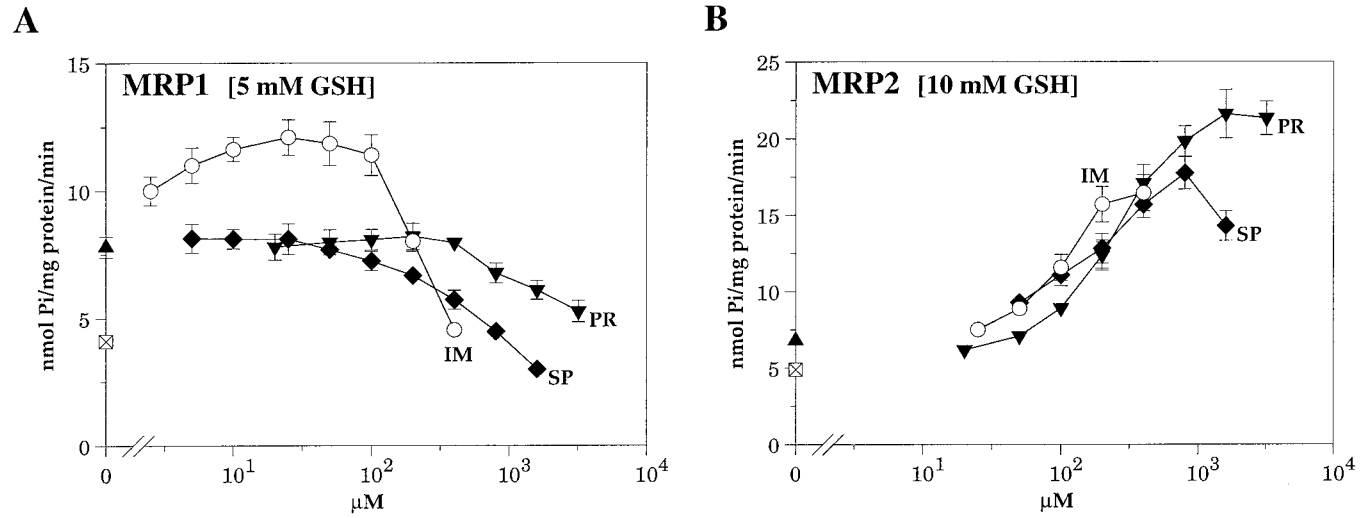


**Fig. 6.** Vanadate-sensitive ATPase activity of isolated *Sf*9 cell membranes expressing MRP1 (A) or MRP2 (B). ATPase activity was measured in the presence of 5 mM GSH (MRP1) or 10 mM GSH (MRP2), and that of probenecid (PR, ▼), sulfinpyrazone (SP, ♦), and indomethacin (IM, ○), for 60 min at 37°C, in the presence of 4 mM ATP (control, ⊠) as described in *Experimental Procedures*. The effect of GSH in the absence of other compounds (▲) is also labeled.

tem provides correct protein folding and insertion into a membrane environment, and the insect cell membranes lack closely related modulator or transporter proteins. The applicability of this system for the study of various multidrug transporters has been demonstrated in several studies (Sarkadi et al., 1992; Bakos et al., 1996, 1998; Gao et al., 1996; Germann, 1998; van Aubel et al., 1998). In this study, we used the baculovirus-*Sf*9 system for the expression of MRP1 and MRP2 and obtained comparable high-level functional expression for both proteins. Because we could not determine the exact level of MRP1/MRP2 expression, the absolute values of the transport or ATPase measurements should be compared with care.

The direct vesicular tracer uptake experiments, as detailed in *Results* and presented in Fig. 2, strongly suggest that both MRP1 and MRP2 can efficiently transport the GS conjugates $LTC_4$ and NEM-GS, although the affinity of MRP1 for these compounds is about one order of magnitude greater than that of MRP2. These results provide a direct comparison of the two proteins in the same membrane environment and reinforce former results obtained for the transport of $LTC_4$ and other GS and glucuronide conjugates in mammalian expression systems (see Jedlitschky et al., 1994, 1996, 1997; Zaman et al., 1995; Cui et al., 1999). When measuring labeled methotrexate uptake, we found that MRP2 had a significantly higher transport capacity for this organic anion than MRP1 (in the case of MRP1, due to the low rate of tracer uptake, we could not properly determine the $K_{1/2}$ values). According to most recent data (Hooijberg et al., 1999), an ATP-dependent methotrexate uptake was also observed in membrane vesicles prepared from both MRP1- and MRP2-transfected human cells.

In transport experiments with labeled NEM-GS, we did not find any major effect of GSH for either MRP1 or MRP2. When examining the effects of various monovalent organic anions on the ATP-dependent NEM-GS uptake (Fig. 3), we found that probenecid effectively inhibited NEM-GS uptake by both MRP1 and MRP2. However, low concentrations of sulfinpyrazone and indomethacin significantly stimulated MRP2-dependent NEM-GS uptake, whereas in the case of MRP1, sulfinpyrazone inhibited and only indomethacin produced such a stimulation. These tracer uptake experiments clearly suggested that various organic anions acted differently on the two transporters and warranted further detailed studies for these interactions.

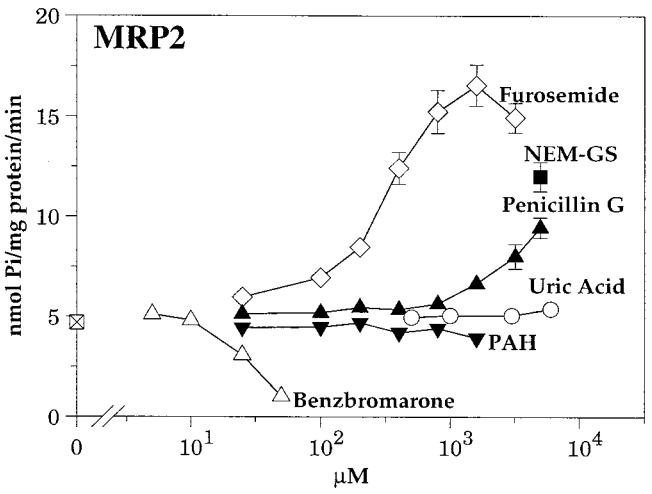


**Fig. 7.** Vanadate-sensitive ATPase activity of isolated *Sf*9 cell membranes expressing MRP2. Effects of furosemide (◇), penicillin G (▲), benzbromarone (△), uric acid (○), and *para*-aminohippuric acid (PAH, ▼) are shown. Membrane ATPase activity was measured for 60 min at 37°C in the presence of 4 mM ATP (control, ⊠), as described in *Experimental Procedures*. The ATPase activity obtained in the presence of 5 mM NEM-GS (■) is also marked.

An efficient and relatively simple way to study substrate interactions with the multidrug transporters is to measure their vanadate-sensitive ATPase activity, either in the original membrane environment (see Sarkadi et al., 1992; Scarborough, 1995) or in isolated and reconstituted systems (Ambudkar et al., 1992). This ATPase activity has been convincingly documented to reflect the turnover rate of these transporters, in which substrate transport is strongly coupled to substrate-stimulated ATPase activity (see Germann, 1998). A low-level, GS conjugate- and flavonoid-stimulated ATPase activity in MRP1-containing mammalian cell membranes has already been noted (Hooijberg et al., 1997), and the isolated and reconstituted MRP1 protein has also been reported to be activated (to about 30–50%) by $LTC_4$ and ADP (Chang et al., 1998). While this manuscript was under revision, Hagmann et al. (1999) reported a substrate-stimulated ATPase activity of the purified and reconstituted MRP2 protein.

As documented in Figs. 4 to 7, vanadate-sensitive ATPase activity measurements in *Sf*9 cell membranes could be efficiently applied to the characterization of the substrate interactions with MRP1 and MRP2. In the isolated *Sf*9 cell membranes, expressing high levels of these exogenous proteins, the transport of NEM-GS or methotrexate was found to closely correlate with the ATPase activity stimulated by these substrates. Moreover, in the case of several substrates, the estimated level of drug stimulation resulted in a 6- to 10-fold increase in the ATPase activity. Based on these results, the MRP-ATPase activity assay can be used to study a large variety of substrate interactions, even if radiolabeled compounds are unavailable or unsuitable for vesicular transport studies.

The most significant differences between MRP1 and MRP2, as observed in both the transport and ATPase experiments, were their interactions with several organic anions, which have already been indicated to inhibit the function of MRP1; these included probenecid, sulfinpyrazone, benzbromarone, and indomethacin (Jedlitschky et al., 1994; Versantwoort et al., 1995; Holló et al., 1996), and their effects on MRP2 have also been noted (Evers et al., 1998).

In human pharmacology, a large number of amphiphilic organic anions are known to bind to albumin in the blood plasma, and although little filtration occurs in the glomeruli, they are actively secreted into both the bile and the lumen of the kidney proximal tubules (see Paulusma et al., 1996; Roch-Ramel, 1998). Here, we studied the effects of such organic anions, including methotrexate, a major anticancer and immunosuppressive drug; the uricosuric compounds probenecid and sulfinpyrazone, which in turn modulate the secretion of many other organic anion antibiotics (e.g., penicillins, cephalosporins, or sulfonamides); indomethacin, an example of a nonsteroid anti-inflammatory agent; furosemide, a widely used diuretic; and the antibiotic penicillin G.

As documented in *Results*, these organic anions predominantly inhibited GS conjugate transport and the ATPase

activity of MRP1 (although a stimulation of MRP1-dependent transport and ATPase was observed by indomethacin). In contrast, the ATPase activity of MRP2 was efficiently activated by all of the above compounds, and sulfinpyrazone, indomethacin, and penicillin G also effectively stimulated MRP2-dependent NEM-GS transport. The effects of GSH or NEM-GS were mostly additive in the ATPase experiments. Extending previous studies, our experiments strongly suggest that MRP1 and MRP2 have different specificities in the transport of organic anions. They also prompt the challenging suggestion that MRP2, in addition to its established function in the liver, plays a key role in the active secretion of organic acid pharmacons in other tissues, such as in the kidney proximal tubules.

In the present study, the effects of GSH on MRP1 and MRP2, respectively, also showed some basic differences. Although MRP1-ATPase was efficiently activated by GSH concentrations corresponding to the cellular levels of this peptide (2–10 mM), MRP2-ATPase was much less sensitive to GSH. $LTC_4$, NEM-GS, or methotrexate transport did not require the presence of GSH in the case of either MRP1 or MRP2, and no significant effect of GSH could be observed on the rate of NEM-GS uptake. Still, an additive effect of GSH on both MRP1 and MRP2 ATPase activities with sulfinpyrazone or probenecid and a synergistic stimulation of the MRP1 ATPase by indomethacin and GSH were observed.

Concerning the role of MRP1 and MRP2 in cytostatic drug resistance, it has been documented in several experiments that both proteins are able to transport unconjugated hydrophobic drugs and anions (Feller et al., 1995; Versantvoort et al., 1995; Holló et al., 1996), but GSH modifies this transport, most likely via a cotransport mechanism (Loe et al., 1996b, 1998; Deeley and Cole, 1997; Evers et al., 1998). In experiments to be reported elsewhere, we found that vinblastine and GSH produced a synergistic stimulation of the MRP2-ATPase (manuscript under preparation).

All of these results suggest a combined, or at least interactively modulated, ATP-dependent transport of GSH and other MRP substrates, and these interactions seem to be different at various GSH and other substrate concentrations. Based on the present results, the question of the mechanistic features of these interactions cannot be properly addressed. Still, all of these data, especially the activation of NEM-GS transport by various monovalent organic anions, strongly suggest the presence of multiple and cooperative drug-binding sites in both MRPs studied. Various cotransport or allosteric activation models should be tested in further, similar, but more elaborate experiments.

In summary, we efficiently applied the *Sf*9 cell membrane expression of MRP1 and MRP2 to compare the transport and ATPase properties of these two proteins and found significant differences in their substrate interactions. The test system used here should allow the examination of a large variety of pharmacologically important compounds to estimate their interactions with these promiscuous transporters. Based on direct transport studies or substrate-stimulated ATPase measurements, we suggest that anionic compounds like methotrexate, probenecid, sulfinpyrazone, furosemide, indomethacin, and penicillin G are actively transported by MRP2, and this transport may have important relevance to the physiological elimination of these widely used therapeutic agents in the liver and the kidney. The observed transport properties of MRP1 and MRP2 should also be considered when devising or applying various inhibitors of tumor cell drug resistance, evoked by the overexpression of one or the other of these proteins.

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