0026-895X/05/6702-545–557$20.00
MOLECULAR PHARMACOLOGY
Vol. 67, No. 2
7138/1192670
Mol Pharmacol 67:545–557, 2005
Printed in U.S.A.

Transport of Bile Acids, Sulfated Steroids, Estradiol 17-β-D-Glucuronide, and Leukotriene C4 by Human Multidrug Resistance Protein 8 (ABCC11)

Zhe-Sheng Chen,[1] Yanping Guo, Martin G. Belinsky, Elena Kotova, and Gary D. Kruh

Medical Science Division, Fox Chase Cancer Center, Philadelphia, Pennsylvania

Received September 13, 2004; accepted November 8, 2004

ABSTRACT

We previously determined that expression of human multidrug resistance protein (MRP) 8, a recently described member of the MRP family of ATP-binding cassette transporters, enhances cellular extrusion of cyclic nucleotides and confers resistance to nucleotide analogs (*J Biol Chem* **278:**29509–29514, 2003). However, the in vitro transport characteristics of the pump have not been determined. In this study, the substrate selectivity and biochemical activity of MRP8 is investigated using membrane vesicles prepared from LLC-PK1 cells transfected with MRP8 expression vector. Expression of MRP8 is shown to stimulate the ATP-dependent uptake of a range of physiological and synthetic lipophilic anions, including the glutathione *S*-conjugates leukotriene C4 and dinitrophenyl *S*-glutathione, steroid sulfates such as dehydroepiandrosterone 3-sulfate (DHEAS) and estrone 3-sulfate, glucuronides such as estradiol 17-β-D-glucuronide ($E_2$17βG), the monoanionic bile acids glycocholate and taurocholate, and methotrexate. In addition, MRP8 is competent in the in vitro transport of cAMP and cGMP, in accord with the results of our previously reported cellular studies. DHEAS, $E_2$17βG, and methotrexate were transported with K_m and V_{max} values of 13.0 ± 0.8 μM and 34.9 ± 9.5 pmol/mg/min, 62.9 ± 12 μM and 62.0 ± 5.2 pmol/mg/min, and 957 ± 28 μM and 317 ± 17 pmol/mg/min, respectively. Based upon the stimulatory action of DHEAS on uptake of $E_2$17βG, the attenuation of this effect at high DHEAS concentrations and the lack of reciprocal promotion of DHEAS uptake by $E_2$17βG, a model involving nonreciprocal constructive interactions between some transport substrates is invoked. These results suggest that MRP8 participates in physiological processes involving bile acids, conjugated steroids, and cyclic nucleotides and indicate that the pump has complex interactions with its substrates.

Investigations of members of the MRP family of ATP-binding cassette transporters have revealed a group of energy-dependent efflux pumps that are able to confer resistance to anticancer agents and transport a striking range of structurally diverse amphipathic anions (Kruh and Belinsky, 2003; Haimeur et al., 2004). Despite their conformity with respect to the transport of lipophilic anions, there are differences in the substrate ranges and functions of these pumps. MRP1 is a ubiquitous efflux pump for glutathione and glucuronate conjugates and plays a specific role in the extrusion of leukotriene C4 (LTC_4) from mast cells (Leier et al., 1994; Jedlitschky et al., 1996; Loe et al., 1996). The substrate range of MRP2 is similar to that of MRP1, but the former pump, by virtue of its expression in canalicular (apical) membranes of hepatocytes, is responsible for the extrusion of glutathione, bilirubin glucuronide, and a variety of pharmaceutical agents into the bile (Ito et al., 1998; Cui et al., 1999;

This work was supported in part by National Institutes of Health Grants CA73728 (to G.D.K.) and CA06927 and by an appropriation from the Commonwealth of Pennsylvania. Z.-S.C. is a recipient of a W. J. Avery Fellowship from the Fox Chase Cancer Center. Y.G. received support from Training Grant CA075266.

[1] Current address: College of Pharmacy and Allied Health Professions, St. John's University, New York, NY 11439.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.007138.

ABBREVIATIONS: MRP, multidrug resistance protein (MRP1-MRP8, gene symbols ABCC1–ABCC6, ABCC10, and ABCC11; MOAT-B, MOAT-C, MOAT-D, and MOAT-E are alternative names for MRP4, MRP5, MRP3, and MRP6, respectively); LTC_4, leukotriene C4; GC, glycocholate; TC, taurocholate; MTX, methotrexate; DHEAS, dehydroepiandrosterone 3-sulfate; DNP-SG, *S*-(2,4-dinitrophenyl)glutathione; $E_1$3S, estrone 3-sulfate; $E_2$17βG, 17β-estradiol 17-(β-D-glucuronide); DHEA, dehydroepiandrosterone; E1, estrone; PGE1, prostaglandin E1; PGE2, prostaglandin E2; DHEAG, dehydroepiandrosterone 3-glucuronide; $E_1$3βG, estrone 3-(β-D-glucuronide); E2, 17β-estradiol; $E_2$3βG, 17β-estradiol 3-(β-D-glucuronide); $E_2$3βG17S, 17β-estradiol 3-(β-D-glucuronide) 17-sulfate; $E_2$3S, 17β-estradiol 3-sulfate; $E_2$3S17βG, 17β-estradiol 3-sulfate 17-(β-D-glucuronide); E3, estriol; $E_3$3βG, estriol 3-(β-D-glucuronide); $E_3$3S, estriol 3-sulfate; $E_3$16βG, estriol 16-(β-D-glucuronide); HEK, human embryonic kidney; PBS, phosphate-buffered saline; MK571, 3-[[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoylethylsulfanyl)methylsulfanyl] propionic acid.

Kawabe et al., 1999; Gerk and Vore, 2002). MRP3, in addition to being able to transport glutathione and glucuronate conjugates, is also able to mediate the transport of the monoanionic bile constituents glycocholate (GC) and taurocholate (TC) (Hirohashi et al., 1999, 2000; Zeng et al., 2000). MRP4 and MRP5, which are structurally distinct from MRP1, MRP2, and MRP3, in that MRP4 and MRP5 possess two membrane spanning domains, whereas the latter proteins have three, have somewhat distinct substrate ranges (Belinsky et al., 1998). These two pumps are able to transport cAMP and cGMP (Jedlitschky et al., 2000; Chen et al., 2001; van Aubel et al., 2002; Wielinga et al., 2003), whereas cyclic nucleotides are not known to be transport substrates of the larger members of the family. Whereas MRP4 and MRP5 have capabilities that are not possessed by MRP1, MRP2, and MRP3, in certain respects the substrate range of at least MRP4 overlaps with those of the latter pumps. As is the case for MRP1, MRP2, and MRP3, MRP4 is able to mediate the transport of glutathione and glucuronate conjugates, folates, and methotrexate (MTX) (Chen et al., 2001, 2002). In addition, like MRP1, MRP4 is able to transport certain sulfated steroids, such as dehydroepiandrosterone 3-sulfate (DHEAS) and estrone 3-sulfate ($E_1$3S) (Qian et al., 2001; Zelcer et al., 2003b), and like MRP3, MRP4 has the facility for transporting monoanionic bile acids (Rius et al., 2003). Reports on MRP6 and MRP7, both of which possess three membrane spanning domains, indicate that MRP6 is able to transport glutathione conjugates and certain amphipathic cyclopentapeptides, and MRP7 has the facility for transporting glucuronate and possibly glutathione conjugates (Madon et al., 2000; Belinsky et al., 2002; Ilias et al., 2002; Chen et al., 2003a).

Accumulating evidence indicates that certain MRPs have cooperative interactions with some of their substrates that may be consequent to the presence of multiple binding sites. The potential for MRPs to be activated in this manner was first raised in the context of the plant MRP AtMRP2, for which mutual stimulation by *S*-(2,4-dinitrophenyl)glutathione (DNP-SG) and $E_2$17βG was described in a detailed study in which it was concluded that this activity was attributable to a mechanism other than cotransport from a common substrate binding site (Liu et al., 2001). More recent studies on mammalian MRPs provided additional support for cooperative interactions. MRP2-mediated transport of $E_2$17βG and bile acids was reported to exhibit mutual stimulation, and $E_2$17βG transport was also subject to stimulation by a range of compounds, including sulfanitran and sulfinpyrazone (Bodo et al., 2003; Zelcer et al., 2003a). In addition, MRP3-mediated transport of $E_2$17βG was reported to be stimulated by the sulfate conjugates of E3040, a benzothiazole derivative, and ethinylestradiol, a synthetic estrogen (Akita et al., 2002; Chu et al., 2004). Furthermore, in several instances, the agents that stimulated transport by MRP2 and MRP3 were not themselves susceptible to transport. Although the preponderance of evidence for how glutathione stimulates transport of uncharged lipophiles such as vincristine supports a working model involving cotransport from a common bipartite binding site composed of separate interacting surfaces for lipophilic and anionic moieties (for review, see Kruh and Belinsky, 2003), it has more recently been appreciated that glutathione can also stimulate transport of lipophilic anions, such as $E_1$3S and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol-glucuronide in the case of MRP1 and GC in the case of MRP4 (Leslie et al., 2001; Rius et al., 2003). This suggests the possibility that glutathione may have an additional binding site in MRP1 and MRP4 that is topologically distinct from the transport substrate binding pocket.

MRP8 is a recently identified MRP family member whose structure resembles MRP4 and MRP5 with respect to possessing only two membrane spanning domains (Bera et al., 2001; Tammur et al., 2001; Yabuuchi et al., 2001). We previously determined that ectopic expression of MRP8 in LLC-PK1 cells enhances cellular efflux of cyclic nucleotides and confers resistance to certain anticancer and antiviral nucleotide analogs (Guo et al., 2003). However, the substrate range of the pump has not been determined and its potential physiological functions are largely unknown. Here, we investigate the substrate selectivity and biochemical activity of MRP8 using membrane vesicles prepared from LLC-PK1 cells transfected with MRP8 expression vector. It is shown that MRP8 is not only able to catalyze the ATP-energized transport of cyclic nucleotides but also that it is able to mediate the transport of a range of lipophilic anions, including the glutathione conjugate LTC_4, sulfated steroids such as DHEAS and $E_1$3S, glucuronides such as 17β-estradiol 17-(β-D-glucuronide) ($E_2$17βG), the bile constituents GC and TC, and monoglutamates such as MTX. Based upon the stimulatory action of DHEAS on uptake of $E_2$17βG, the attenuation of this effect at high DHEAS concentrations and the lack of reciprocal promotion of DHEAS uptake by $E_2$17βG, a model involving nonreciprocal constructive interactions between some transport substrates is invoked. These results suggest that MRP8 is involved in physiological processes involving bile acids, conjugated steroids, and cyclic nucleotides and indicate that the pump has complex interactions with its substrates.

Materials and Methods

Materials. [^{3}H]$E_2$17βG (45.0 Ci/mmol), [^{3}H]dehydroepiandrosterone (DHEA; 74.0 Ci/mmol), [^{3}H]DHEAS (74.0 Ci/mmol), [^{3}H]LTC_4 (130 Ci/mmol), [^{3}H]estrone (E1; 65 Ci/mmol), [^{3}H]$E_1$3S (46 Ci/mmol), [^{3}H]TC (2.0 Ci/mmol), and [^{14}C]chenodeoxycholic acid (0.049 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{3}H]cGMP (6.8 Ci/mmol), [^{3}H]cAMP (17 Ci/mmol), [^{3}H]MTX (23 Ci/mmol), [^{3}H]MTX-Glu2 (15 Ci/mmol), [^{3}H]MTX-Glu3 (17 Ci/mmol), and [^{3}H]folic acid (42 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). [^{14}C]GC (0.056 Ci/mmol), [^{3}H]prostaglandin E1 (PGE1; 48 Ci/mmol), and [^{3}H]prostaglandin E2 (PGE2; 187 Ci/mmol) were purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). [^{3}H]Cholic acid (25 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Creatine kinase, creatine phosphate, ATP, AMP, 1-chloro-2,4-dinitrobenzene, unlabeled $E_2$17βG, LTC_4, cGMP, cAMP, DHEA, dehydroepiandrosterone 3-glucuronide (DHEAG), DHEAS, E1, $E_1$3S, GC, TC, chenodeoxycholate, folic acid, PGE1, PGE2, estrone 3-(β-D-glucuronide) $E_1$3βG, $E_1$3S 17β-estradiol (E2); 17β-estradiol 3-(β-D-glucuronide) ($E_2$3βG), 17β-estradiol 3-(β-D-glucuronide) 17-sulfate ($E_2$3βG17S), 17β-estradiol 3-sulfate ($E_2$3S), 17β-estradiol 3-sulfate 17-(β-D-glucuronide) ($E_2$3S17βG), estriol (E3), estriol 3-(β-D-glucuronide) ($E_3$3βG), estriol 3-sulfate ($E_3$3S), and estriol

16-(β-D-glucuronide) ($E_3$16βG) were purchased from Sigma-Aldrich (St. Louis, MO). Unlabeled MTX and MTX polyglutamates were purchased from Schircks Laboratories (Jona, Switzerland). DNP-SG and [^{3}H]DNP-SG were synthesized from 1-chloro-2,4-dinitrobenzene and unlabeled or labeled [^{3}H]glycine-2-glutathione (44.8 Ci/mmol; PerkinElmer Life and Analytical Sciences), as described previously (Awasthi et al., 1981). LLC-PK1-MRP8-3 and control LLC-PK1-pcDNA cells were described previously (Guo et al., 2003). HEK293 cells transduced with MRP4 and control HEK293 cells were kindly provided by Piet Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). LLC-PK1 and HEK293 cells, respectively, were grown in M-199 medium or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine.

Preparation of Membrane Vesicles. Membrane vesicles were prepared by the nitrogen cavitation method as described previously (Cornwell et al., 1986). Cells were washed with PBS and then scraped into PBS containing 1% aprotinin. Cells were then washed at 4°C in PBS, collected by centrifugation (4000*g* for 10 min), suspended in buffer A (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM *p*-amindinophenylmethanesulfonylfluoride, and 0.2 mM $CaCl_2$) and equilibrated at 4°C for 15 min under a nitrogen pressure of 500 psi. EDTA was added to the suspension of lysed cells to a final concentration of 1 mM, and the suspension was then diluted 1:4 with buffer B (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, and 1 mM *p*-amindinophenylmethanesulfonylfluoride) and centrifuged at 4000*g* for 10 min at 4°C to remove nuclei and unlysed cells. The supernatant was layered onto a sucrose cushion (35% sucrose, 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA) and centrifuged for 30 min at 16,000*g* at 4°C. The interface was collected and centrifuged for 45 min at 100,000*g* for 4°C. The pellet was resuspended in buffer B by repeated passage through a 25-gauge needle. Protein concentration was determined by the method of Bradford (Bradford and Ward, 1976). Vesicles were stored at −80°C.

Immunoblot Analysis. Membrane vesicle preparations were resolved by 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters using a wet transfer system, as described previously (Laemmli, 1970; Towbin et al., 1979). MRP8 was detected using previously described polyclonal anti-MRP8 antibody at a dilution of 1:500 and alkaline phosphatase-conjugated secondary antibody (Guo et al., 2003).

In Vitro Transport Assays. Transport assays were performed using the rapid filtration method essentially as described previously (Leier et al., 1994). Assays were carried out at 37°C in medium containing membrane vesicles (10 μg), 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 4 mM ATP or 4 mM AMP, 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase, and radiolabeled substrate ± unlabeled substrate, in a total volume of 50 μl. Reactions were carried out at 37°C and stopped by the addition of 3 ml of ice-cold stop solution (0.25 M sucrose, 100 mM NaCl, and 10 mM Tris-HCl, pH 7.4). For the rapid filtration step samples were passed through 0.22-μm GVWP filters (Millipore Corporation, Billerica, MA) presoaked in the stop solution, except for DHEA, DHEAS, E1, and E1S transport assays, for which presoaked glass fiber filters (type A/E; Gelman Sciences, Montreal, PQ, Canada) were used. The filters were washed three times with 3 ml of ice-cold stop solution and dried at room temperature. Radioactivity was measured by the use of a liquid scintillation counter. Rates of net ATP-dependent transport were determined by subtracting the values obtained in the presence of 4 mM AMP from those obtained in the presence of 4 mM ATP. Uptake rates were linear for at least 5 min, and rates for concentration dependence experiments were measured at 5 min.

Data Analysis. Kinetic parameters were computed by nonlinear least-squares analysis (Marquardt, 1963) using Ultrafit computer software (Biosoft, Ferguson, MO).

Results

Expression of MRP8 in Membrane Vesicles Prepared from MRP8-Transfected LLC–PK1 Cells. MRP8-dependent transport activity was assayed on density-fractionated membrane vesicles prepared from previously described LLC-PK1 cells transfected with MRP8 expression vector (LLC-PK1-MRP8-3) and parental plasmid-transfected control cells (LLC-PK1-pcDNA) (Guo et al., 2003). As determined by immunoblot analysis, LLC-PK1-MRP8-3 membranes were a rich source of MRP8 protein, which migrates as a 170,000- to 190,000-Da electrophoretic species (Fig. 1). This apparent molecular mass is larger than the calculated molecular mass of MRP8 (~154,000 Da), as would be expected for a glycosylated transmembrane protein.

Transport of cAMP and cGMP by MRP8. To determine whether experiments using LLC-PK1-MRP8-3 membrane vesicle preparations faithfully reflected the transport characteristics of the pump, uptake of cAMP and cGMP, compounds we had inferred previously from cellular measurements to be MRP8 transport substrates, were examined in the first instance (Guo et al., 2003). As expected, MRP8-enriched membrane vesicles catalyzed enhanced energy-dependent transport of both of these cyclic nucleotides (Fig. 2). At an initial concentration of 1 μM [^{3}H]cAMP, uptake by MRP8 membrane vesicles in the presence of ATP was 0.74 pmol/mg/min when measured at the 5-min time point of the assay, whereas the values for the same vesicles in the presence of AMP or by the control vesicles in the presence of either ATP or AMP, were less than 0.53 pmol/mg/min. The corresponding values for uptake of 1 μM [^{3}H]cGMP were 0.59 pmol/mg/min and less than 0.39 pmol/mg/min. MRP8-mediated transport of cAMP and cGMP was attenuated by increasing the sucrose concentration in the transport buffer, indicating that the substrates were delivered into an osmotically sensitive compartment (data not shown).

Transport of Glutathione and Glucuronate Conjugates, and Monoanionic Bile Acids by MRP8. The facility of MRP8 for transporting a variety of amphipathic anions that are established transport substrates of various MRP family members was examined next (representative structures are shown in Fig. 3). LTC_4, DNP-SG, and $E_2$17βG were used as model test compounds for glutathione and glucuronate conjugates. These compounds were all subject to MRP8-mediated transport (Fig. 4, A–C). When measured at an initial concentration of 20 nM, [^{3}H]LTC_4 was transported

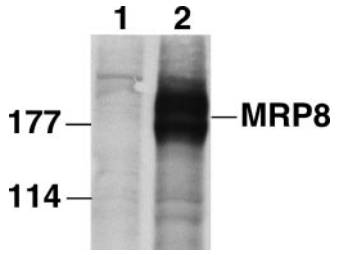

Fig. 1. Immunoblot detection of MRP8 in membrane vesicles prepared from MRP8-transfected LLC-PK1 cells. Membrane vesicles (10 μg) were prepared from MRP8-transfected (lane 2, LLC-PK1-MRP8-3) or parental vector-transfected (lane 1, LLC-PK1-pcDNA) LLC-PK1 cells. Proteins were resolved by SDS-polyacrylamide gel electrophoresis on 8% gels, electrotransferred to nitrocellulose membranes, and incubated with anti-MRP8 polyclonal antibody. The sizes of molecular mass standards (in kilodaltons) and the location of MRP8 are indicated.

at a rate of 0.42 pmol/mg/min by LLC-PK1-MRP8-3 membranes in the presence of ATP. By comparison, the rates by the same membranes in the presence of AMP, or by the control membranes in the presence of ATP or AMP, were 0.24, 0.26, and 0.21 pmol/mg/min, respectively. [^{3}H]DNP-SG at an initial concentration of 20 μM was transported at a rate of 15.9 pmol/mg/min by MRP8-enriched membranes in the presence of ATP, but at less than 8.6 pmol/mg/min by the same membranes in the presence of AMP, or by the control membranes in the presence ATP or AMP. ATP-energized transport of 1 μM [^{3}H]$E_2$17βG by MRP8-enriched was 4.8 pmol/mg/min, by comparison with only 1.2 pmol/mg/min for the same membranes in the presence of AMP. ATP-dependent transport of [^{3}H]$E_2$17βG was also detected for the control membranes in the presence of ATP, but at rates (2.2 pmol/mg/min) that were consistently lower than that of the MRP8-enriched membranes. Transport of the monoanionic bile acids [^{14}C]GC and [^{3}H]TC were also stimulated by MRP8 (Fig. 4, D and E). [^{14}C]GC, at an initial concentration of 100 μM, was taken up at rates of 177, 105, 94.3, and 88.7 pmol/mg/min, respectively, by MRP8-enriched membrane vesicles in the presence of ATP and AMP, and by control vesicles in the presence of ATP and AMP. The corresponding values for [^{3}H]TC were 23.0, 14.4, 13.0, and 11.1 pmol/mg/min, respectively. By contrast with the two conjugated bile acids, MRP8 was unable to transport [^{3}H]cholic acid, the unconjugated parent compound of GC and TC, or [^{14}C]chenodeoxycholic acid, another unconjugated bile acid (data not shown).

Transport of Sulfated Steroids. The ability of MRP8 to transport sulfated steroids was examined next using DHEAS and $E_1$3S as model test compounds. Both of these compounds were subject to MRP8-mediated transport (Fig. 5, A and C).

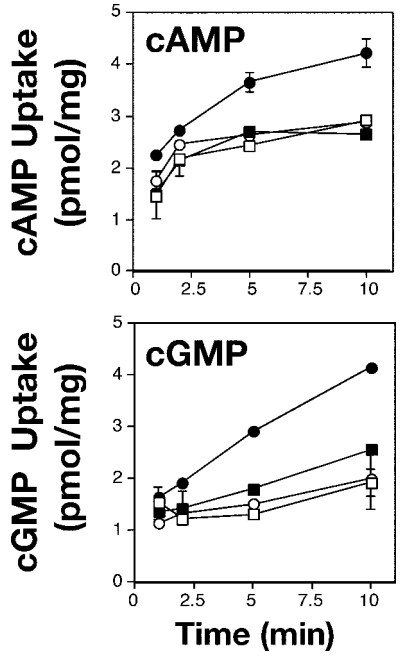

Fig. 2. Time course of ATP-dependent uptake of [^{3}H]cAMP and [^{3}H]cGMP into membrane vesicles. Membrane vesicles prepared from LLC-PK1-MRP8-3 (circles) or LLC-PK1-pcDNA (squares) were incubated at 37°C in uptake medium containing 1 μM [^{3}H]cAMP (A) or 1 μM [^{3}H]cGMP (B). Closed symbols, uptake from medium containing 4 mM MgATP; open symbols, uptake from medium containing 4 mM MgAMP. The values shown are means ± S.E. for a representative experiment performed in duplicate.

[^{3}H]DHEAS at an initial concentration of 100 nM was taken up by LLC-PK1-MRP8-3 membrane vesicles at rate of 1.0 pmol/mg/min from media containing ATP, and at rate of only 0.42 pmol/mg/min from media containing AMP (Fig. 5A). Similar to the case with $E_2$17βG, ATP-energized transport of DHEAS was detected for the control membranes, but at rates that were consistently lower than those of MRP8-enriched membranes. For control membranes the uptake rates were 0.61 and 0.35 pmol/mg/min from media containing ATP and AMP, respectively. MRP8-mediated uptake of 100 μM [^{3}H]DHEA was not observed to any extent, indicating that transport of [^{3}H]DHEAS was dependent upon its covalent modification with a 3′ sulfate moiety (Fig. 5B). The transport characteristics of MRP8 with respect to $E_1$3S paralleled the situation for DHEAS, in that whereas MRP8-mediated uptake of [^{3}H]$E_1$3S was readily apparent, uptake of the unmodified parent compound [^{3}H]E1 was undetectable (Fig. 5, C and D). Uptake of 1 μM [^{3}H]$E_1$3S by LLC-PK1-MRP8-3 membranes was 2.8 pmol/mg/min under energized conditions, by contrast with rates of less than 2.2 pmol/mg/min for the same membranes in media containing AMP or control membranes in media containing either ATP or AMP.

Analysis of Prostaglandin E1 and Prostaglandin E2 Transport by MRP8. Recently, MRP4 was reported to be competent in the transport of PGE1 and PGE2 (Reid et al., 2003). By contrast with MRP4, MRP8 was unable to transport either of these compounds (Fig. 6). Under assay conditions in which uptake of 100 nM [^{3}H]PGE1 or 10 nM

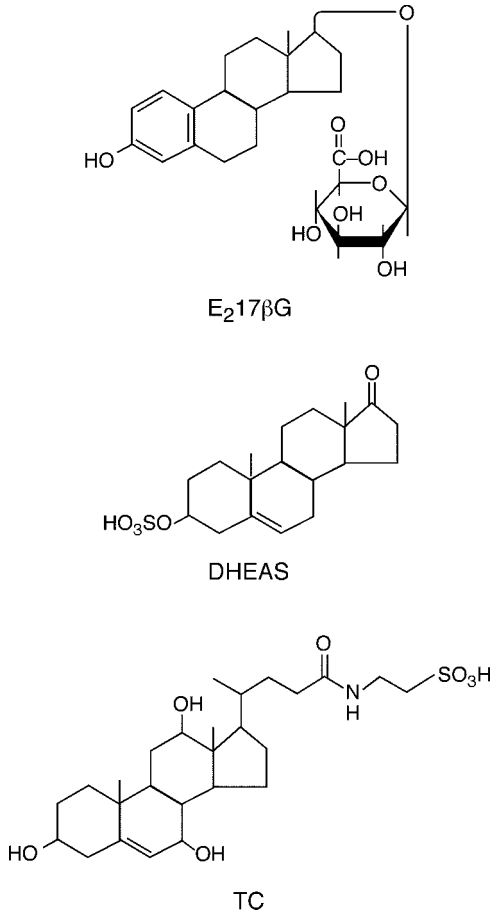

Fig. 3. Structures of representative compounds mentioned in the text. The structures of $E_2$17βG, DHEAS, and TC are shown.

[^{3}H]PGE2 was readily observed for membrane vesicles prepared from HEK293 cells transduced with MRP4, MRP8-dependent transport was undetectable.

Transport of MTX by MRP8. The ability of MRP8 to transport MTX, a monoglutamate and folic acid analog, was examined next. Robust MRP8-mediated uptake of this antifolate was observed (Fig. 7A). The uptake rate for 100 μM [^{3}H]MTX by LLC-PK1-MRP8-3 membranes under energized conditions was 106 pmol/mg/min, compared to rates of less than 49.2 pmol/mg/min for the same membranes under non-energized conditions, or the control membranes in the presence of either ATP or AMP. By contrast with transport of [^{3}H]MTX, MRP8-mediated uptake of 100 μM concentrations of either [^{3}H]MTX-Glu2 and [^{3}H]MTX-Glu3 was negligible (Fig. 7B). ATP-dependent uptake attributable to MRP8 was 405 pmol/mg/10 min, whereas the corresponding value for [^{3}H]MTX-Glu2 was 15 pmol/mg/10 min, and MRP8-dependent uptake of [^{3}H]MTX-Glu3 was undetectable. As expected, MRP8 was able to mediate the transport of folic acid, albeit at lower levels than observed for MTX (Fig. 7B).

Kinetics of $E_2$17βG, DHEAS, and MTX Uptake by MRP8. The concentration dependence of [^{3}H]$E_2$17βG,

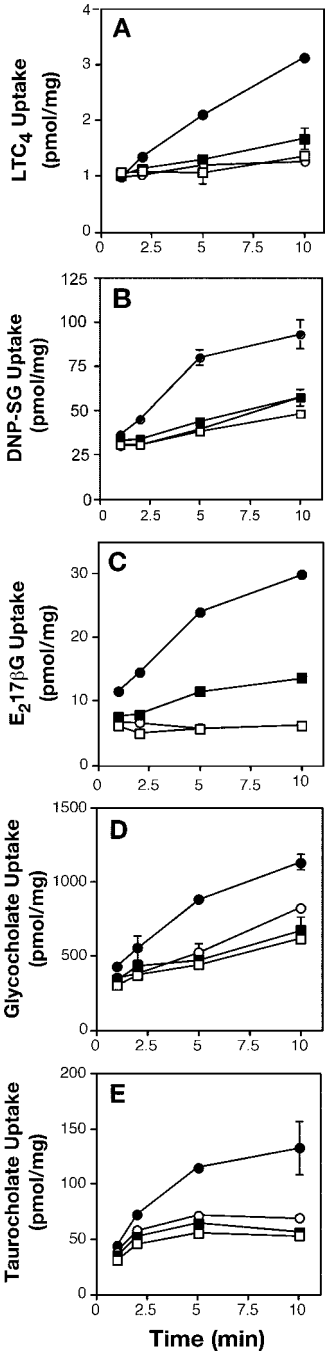

Fig. 4. Time course of ATP-dependent uptake of [^{3}H]LTC_4, [^{3}H]DNP-SG, [^{3}H]$E_2$17βG, [^{14}C]glycocholate, and [^{3}H]taurocholate into membrane vesicles. Membrane vesicles prepared from LLC-PK1-MRP8-3 (circles) or LLC-PK1-pcDNA (squares) were incubated at 37°C in uptake medium containing 20 nM [^{3}H]LTC_4 (A), 20 μM [^{3}H]DNP-SG (B), 1 μM [^{3}H]$E_2$17βG (C), 100 μM [^{14}C]glycocholate (D), or 10 μM [^{3}H]taurocholate (E). Closed symbols, uptake from medium containing 4 mM MgATP; open symbols, uptake from medium containing 4 mM MgAMP. The values shown are means ± S.E. for a representative experiment performed in duplicate.

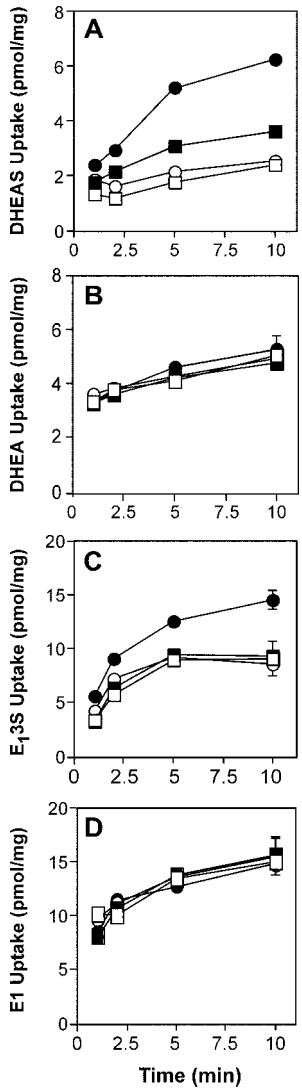

Fig. 5. Time course of ATP-dependent uptake of [^{3}H]DHEAS, [^{3}H]DHEA, [^{3}H]E1S, and [^{3}H]E1 into membrane vesicles. Membrane vesicles prepared from LLC-PK1-MRP8-3 (circles) or LLC-PK1-pcDNA (squares) were incubated at 37°C in uptake medium containing 100 nM [^{3}H]DHEAS (A), 100 nM [^{3}H]DHEA (B), 1 μM [^{3}H]E1S (C), or 1 μM [^{3}H]E1 (D). Closed symbols, uptake from medium containing 4 mM MgATP; open symbols, uptake from medium containing 4 mM MgAMP. The values shown are means ± S.E. of a representative experiment performed in duplicate.

[^{3}H]DHEAS, and [^{3}H]MTX uptake was analyzed. The substrate concentration dependence of ATP-energized [^{3}H]$E_2$17βG and [^{3}H]DHEAS transport by MRP8-enriched membranes and control membranes approximated Michaelis-Menten kinetics (Figs. 8 and 9). For [^{3}H]$E_2$17βG, nonlinear least-squares fitting of the data to the Michaelis-Menten equation for three independence determinations yielded K_m and V_{max} values for MRP8-enriched membrane vesicles and control membrane vesicles of 58.3 ± 6.5 μM and 100.8 ± 3.2 pmol/mg/min, and 70.9 ± 16.3 μM and 44.4 ± 4.0 pmol/mg/min, respectively (Fig. 8A). ATP-dependent uptake attributable to MRP8 was enumerated as the difference in the uptake in medium containing ATP between MRP8-enriched membrane vesicles and control membrane vesicles (Fig. 8B). This yielded K_m and V_{max} values of 62.9 ± 12 μM and 62.0 ± 5.2 pmol/mg/min for MRP8 (Fig. 8B). For [^{3}H]DHEAS, the K_m and V_{max} values for MRP8-enriched membrane vesicles and control membrane vesicles were 8.9 ± 3.3 μM and 39.6 ± 4.1 pmol/mg/min, and 7.2 ± 1.3 μM and 16.4 ± 3.7 pmol/mg/min, respectively (n = 3) (Fig. 9A). Analysis of ATP-dependent uptake attributable to MRP8 yielded K_m and V_{max} values of 13.0 ± 0.8 μM and 34.9 ± 9.5 pmol/mg/min (Fig. 9B).

Transport of [^{3}H]MTX also exhibited saturation kinetics (Fig. 10). The K_m and V_{max} values were 957 ± 28 μM and 317 ± 17 pmol/mg/min (n = 3). The efficiencies (V_{max}/K_m ratios) of ATP-dependent uptake therefore fell in the rank order of DHEAS (2.7) > $E_2$17βG (1.0) ≫ MTX (0.30; Table 1).

Effect of Substrates and Other Compounds on $E_2$17βG Transport. To gain insight into the interactions of MRP8 with its substrates and other compounds a range of compounds were screened for their capacity to inhibit $E_2$17βG uptake into MRP8-enriched membrane vesicles. In the first instance, 10 μM concentrations of substrates were analyzed (Table 2). The inhibitions exerted by the majority of these compounds, including cAMP, cGMP, glycocholate, taurocholate, and MTX were moderate (26.2–37.9%). In accord with the finding that MTX, but not MTX polyglutamates, is a transport substrate, the inhibition exerted by MTX-Glu2 (10.9%) was weaker by comparison with the parent compound (30.5%), and inhibition by MTX-Glu3 was not detected to any extent. Of the known substrates tested, DNP-SG (83.4 and 11.6% at 10 and 1 μM, respectively) and LTC_4 (27.7% at 1 μM; LTC_4 concentration is limited by its solubility) were the most potent inhibitors. In contrast to the latter substrates, DHEAS exerted a stimulatory effect (~2-fold) on $E_2$17βG uptake. However, the dianionic bile acid glycolithocholate, which, like DHEAS, bears a 3′ sulfate substituent, not only failed to stimulate transport but instead exerted a

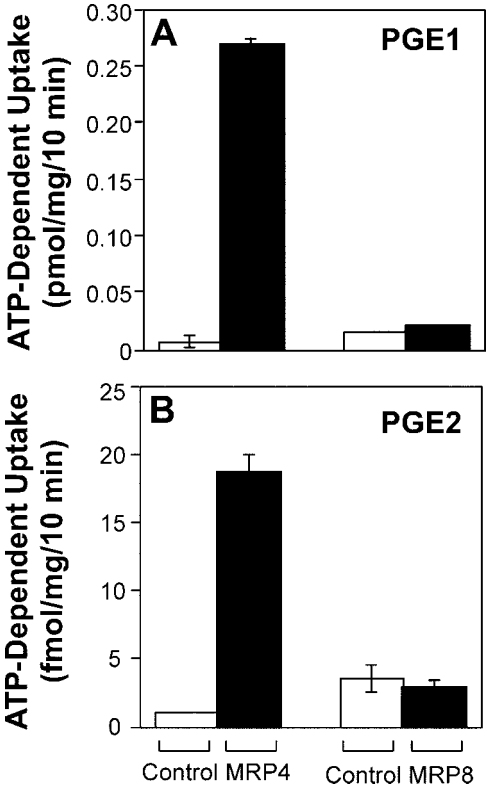

Fig. 6. Analysis of ATP-dependent uptake of [^{3}H]PGE1 and [^{3}H]PGE2 into membrane vesicles. Membrane vesicles prepared from LLC-PK1-MRP8-3, and HEK/MRP4 cells, and their respective parental control cell lines LLC-PK1-pcDNA and HEK293, were incubated at 37°C in uptake medium containing 100 nM [^{3}H]PGE1 (A) or 10 nM [^{3}H]PGE2 (B). The values shown are means ± S.E. for net ATP-dependent uptake (values for uptake in medium containing 4 mM AMP subtracted from values obtained in medium containing 4 mM ATP) of a representative experiment performed in duplicate.

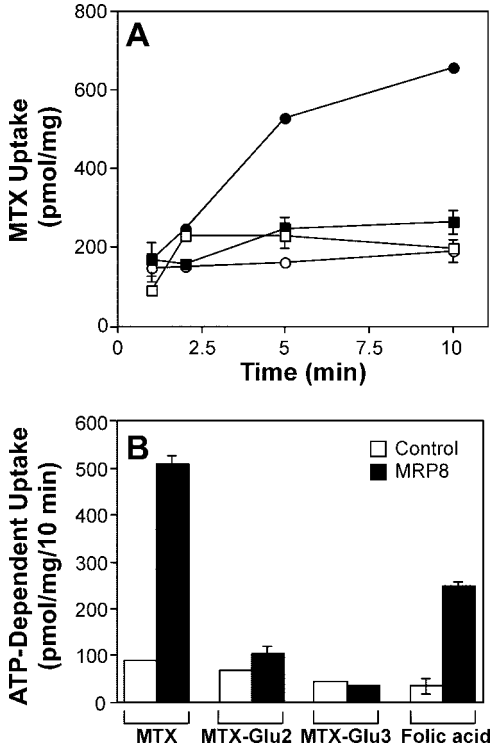

Fig. 7. Analysis of ATP-dependent uptake of MTX, MTX polyglutamates, and folic acid into membrane vesicles. A, time course of ATP-dependent uptake of [^{3}H]MTX into membrane vesicles. Membrane vesicles prepared from LLC-PK1-MRP8-3 (circles) or LLC-PK1-pcDNA (squares) were incubated at 37°C in uptake medium containing 100 μM [^{3}H]MTX. Closed symbols, uptake from medium containing 4 mM MgATP; open symbols, uptake from medium containing 4 mM MgAMP. The values shown are means ± S.E. of a representative experiment performed in duplicate. B, uptake of [^{3}H]MTX, [^{3}H]MTX polyglutamates, and [^{3}H]folic acid into membrane vesicles. Membranes prepared from LLC-PK1-MRP8-3 or LLC-PK1-pcDNA were incubated for 10 min at 37°C in uptake medium containing 100 μM concentrations of [^{3}H]MTX, [^{3}H]MTX-Glu2, [^{3}H]MTX-Glu3, or [^{3}H]folic. The values shown are means ± S.E. for net ATP-dependent uptake (uptake in medium containing 4 mM AMP subtracted from values obtained in medium containing 4 mM ATP) of a representative experiment performed in duplicate.

degree of inhibition (84.1%) comparable with that of DNP-SG and LTC4.

Next, the effects of a range of compounds that are inhibitors of organic anion transporters were examined (Table 2). In accord with the capacity of the glutathione conjugates DNP-SG and LTC4 to inhibit transport, the LTD4 analog MK571 was the most potent of the inhibitors tested (17.7 and 75.0% at 1 and 10 μM concentrations, respectively). The inhibitions exerted by probenecid and indomethacin were moderate (24.4 and 35.9%), and sildenafil and penicillin G were weak inhibitors (21.1 and 8.9%, respectively). By contrast with the inhibitory effects of the later compounds, sulfanitran and sulfinpyrazone stimulated $E_2$17βG uptake into MRP8-enriched vesicles (~1.3- and 1.5-fold, respectively). Neither glucuronic acid nor sodium sulfate exerted appreciable inhibitions, at either 10 μM (Fig. 11A) or 1 mM (data not shown).

The effects of compounds that either did (DHEAS, sulfanitran, and sulfinpyrazone) or did not (penicillin G) stimulate $E_2$17βG uptake into MRP8-enriched membrane vesicles were analyzed in further detail in experiments in which control vesicles and a range of modulator concentrations were used (Fig. 11). These experiments confirmed that DHEAS was able to stimulate MRP8-mediated transport of $E_2$17βG but showed that the effects of sulfanitran and sulfinpyrazone were attributable to stimulation of endogenous transporters present in LLC-PK1 cells.

Stimulation of $E_2$17βG uptake into membranes prepared from LLC-PK1-MRP8-3 cells was apparent at all DHEAS concentrations (10–1000 μM), with the maximum effect observed at 100 μM (Fig. 11A, black columns). Stimulation was also observed for the control membranes, for which the greatest effect (3.6-fold) was observed at 30 μM DHEAS (white columns). However, an increment in uptake consequent to DHEAS and specific for MRP8 was clearly discernable, as indicated by the MRP8-dependent transport values (striped columns), enumerated as the ATP-dependent uptake values for MRP8-enriched membranes minus

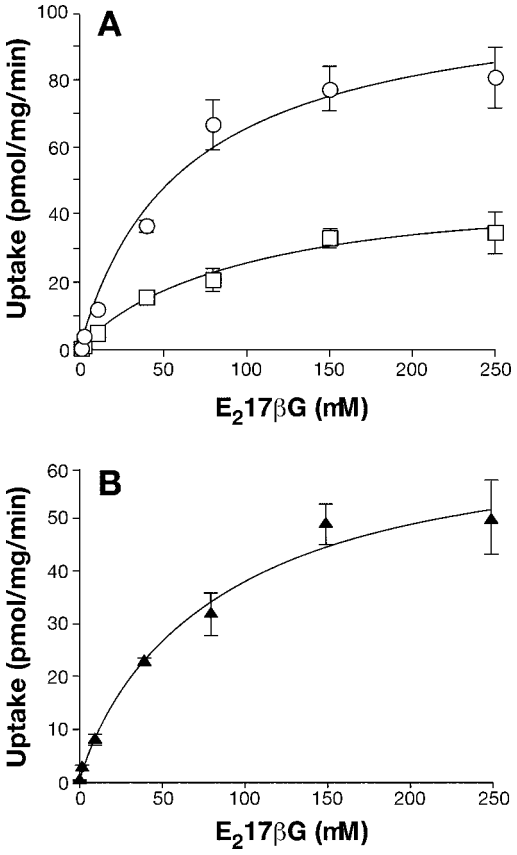

Fig. 8. Kinetics of Transport of $E_2$17βG. A, concentration dependence of [^{3}H]$E_2$17βG uptake by LLC-PK1-MRP8-3 and control membrane vesicles. The rates of ATP-dependent uptake of [^{3}H]$E_2$17βG into membrane vesicles (10 μg) prepared from MRP8 transfected LLC-PK1/MRP8-3 (circles) and from parental plasmid-transfected LLC-PK1-pcDNA cells (squares) were measured for 5 min at 37°C in uptake media containing 4 mM ATP or 4 mM AMP. The values shown (means ± S.E.) are rates measured in the presence of ATP minus rates measured in the presence of AMP for a measurement performed in triplicate. B, concentration dependence of [^{3}H]$E_2$17βG uptake by MRP8. The values shown are derived from A and represent rates measured in the presence of ATP for membrane vesicles from MRP8-transfected LLC-PK1-MRP8-3 cells minus rates measured in the presence of ATP for membrane vesicles from parental plasmid vector transfected LLC-PK1-pcDNA cells. Measurements were performed at least three times, and a representative experiment is shown.

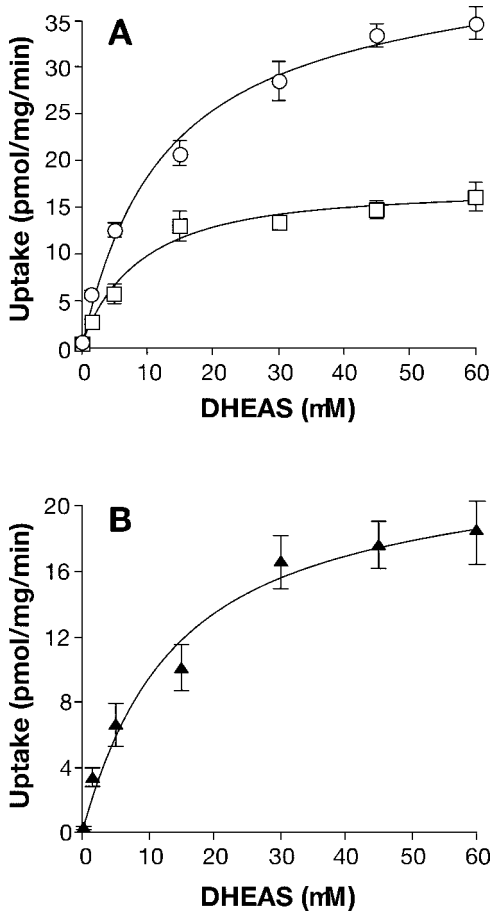

Fig. 9. Kinetics of transport of DHEAS. A, concentration dependence of [^{3}H]DHEAS uptake by LLC-PK1-MRP8-3 and control membrane vesicles. The rates of ATP-dependent uptake of [^{3}H]DHEAS into membrane vesicles (10 μg) prepared from MRP8 transfected LLC-PK1-MRP8-3 (circles) and from parental plasmid-transfected LLC-PK1-pcDNA cells (squares) were measured for 5 min at 37°C in uptake media containing 4 mM ATP or 4 mM AMP. The values shown (means ± S.E.) are rates measured in the presence of ATP minus rates measured in the presence of AMP for a measurement performed in triplicate. B, concentration dependence of [^{3}H]DHEAS uptake by MRP8. The values shown (means ± S.E.) are derived from A and represent rates measured in the presence of ATP for membrane vesicles from MRP8-transfected LLC-PK1-MRP8-3 cells minus rates measured in the presence of ATP for membrane vesicles from LLC-PK1-pcDNA cells. Measurements were performed at least three times and representative experiments are shown.

the ATP-dependent uptake values for the control membranes. At 100 μM DHEAS, MRP8-mediated uptake of $E_2 17\beta G$ was stimulated 5.8-fold. At higher DHEAS concentrations promotion of MRP8-dependent uptake was attenuated. By contrast with DHEAS, sulfanitran and sulfinpyrazone stimulated uptake by control vesicles but had only barely appreciable effects (1.3- and 1.2-fold, respectively, at 10 μM) on MRP8-mediated transport (Fig. 11, B and C). The modest stimulatory effect on MRP8-dependent transport was attenuated at higher concentrations of sulfanitran, and in the case of sulfinpyrazone higher concentrations exerted an inhibitory effect. Penicillin G at a concentration of 10 μM did not affect MRP8-mediated transport, but was a potent inhibitor at 100 and 1000 μM (Fig. 11D).

Next, the dependence of stimulation of $E_2 17\beta G$ transport on covalent modifications of DHEA, as well as the effects of other steroids, were examined (Fig. 12A). In contrast to the stimulation exerted by DHEAS, DHEAG was a potent inhibitor (67.4%) and DHEA did not affect transport to any extent. This suggested that stimulation was dependent upon modification of DHEA with sulfate, and that substitution of sulfate with glucuronic acid yielded an inhibitory compound. A strikingly similar pattern was observed when the effects of covalent modification at the position 3 was evaluated in the context of three other steroids. By comparison with the negligible effects of E1, E2, and E3 on $E_2 17\beta G$ transport, the respective 3′ sulfated compounds, $E_1 3S$, $E_2 3S$, and $E_3 3S$, stimulated transport, and the respective 3′ glucuronidated compounds were all potent inhibitors. $E_1 3S$, $E_2 3S$, and $E_3 3S$ stimulated uptake by 2.1-, 2.7-, and 1.6-fold, respectively, whereas $E_1 3G$, $E_2 3G$, and $E_3 3G$ inhibited transport by 87.9, 86.0, and 78.8%, respectively. Interestingly, modification of $E_2 3S$ with a D ring glucuronic acid moiety at the 17 position ($E_2 3S 17\beta G$) not only abrogated the stimulation associated with the A ring sulfate substituent but also yielded the most potent inhibitor (97.2%) of the series of compounds tested in Fig. 12A. $E_2 3\beta G 17S$, an E2 derivative bearing both sulfate and glucuronic acid modifications, but in which the respective positions of the substituents are reversed compared with $E_2 3S 17\beta G$, was also a potent inhibitor (81.6%), albeit it somewhat less potent that the latter compound, and about as potent as $E_2 3\beta G$. In combination the analysis of the substituted E2 compounds indicated that the presence of either an A or a D ring glucuronate substituent was sufficient to exert an inhibitory effect. In accord with this notion, the D ring glucuronide $E_3 16\beta G$ was also a potent inhibitor (92.1%).

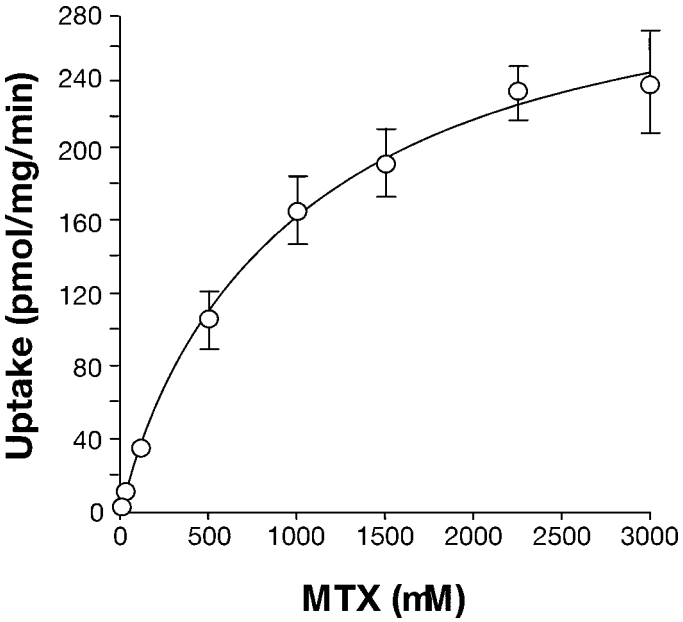

Fig. 10. Kinetics of Transport of MTX. The rates of ATP-dependent uptake of [^{3}H]MTX into membrane vesicles (10 μg) prepared from LLC-PK1-MRP8-3 were measured for 5 min at 37°C in uptake media containing 4 mM ATP or 4 mM AMP. The values shown (means ± S.E.) are rates measured in the presence of ATP minus rates measured in the presence of AMP for a measurement performed in triplicate. Measurements were repeated at least three times, and a representative experiment is shown.

TABLE 1

Summary of kinetic parameters for MRP8-mediated transport

Kinetic parameters were computed as described in the legends to Figs. 7–9 and under *Materials and Methods* Values shown are means ± S.E. of at least three independent measurements performed in triplicate.

Substrate	K_m	V_{max}	V_{max}/K_m
	mM	*pmol/mg/min*	
$E_2 17\beta G$	62.9 ± 12	62.0 ± 5.2	1.0
DHEAS	13.0 ± 0.8	34.9 ± 9.5	2.7
MTX	957 ± 28	317 ± 17	0.30

Effects of Substrates and Other Compounds on DHEAS Transport. The same compounds analyzed in the context of $E_2 17\beta G$ transport were evaluated for their effects on DHEAS uptake by MRP8-enriched membranes (Table 3). Whereas DHEAS was able to stimulate $E_2 17\beta G$ transport (Table 2; Fig. 10A), the reverse was not the case, in that $E_2 17\beta G$ exerted an inhibitory effect on DHEAS transport (19.7%). The inhibitions exerted on DHEAS uptake by the other compounds were similar to the effects observed for the same compounds on $E_2 17\beta G$ transport (Table 2). Thus, LTC_4

TABLE 2

Effect of Compounds on MRP8-mediated transport of [^{3}H]$E_2 17\beta$ G

Membrane vesicles prepared from LLC/PK1-MRP8-3 cells were incubated at 37° C for 10 min in transport media containing 1 μM [^{3}H]$E_2 17\beta$ G in the presence of the indicated compounds. ATP-dependent uptake was calculated by subtracting values obtained in the presence of 4 mM ATP from those in the presence of 4 mM AMP. Transport is expressed as percentage uptake in the absence of modulator. Values shown are means ± S.E. of at least three measurements performed in duplicate.

Compound	Concentration	Uptake
	μM	*% of control*
Control		100
cAMP	10	73.8 ± 2.6
cGMP	10	72.5 ± 2.8
LTC_4	1	72.3 ± 2.0
DNP-SG	1	88.4 ± 5.5
DNP-SG	10	16.6 ± 2.1
MTX	10	69.5 ± 8.4
MTX-Glu2	10	89.1 ± 7.2
MTX-Glu3	10	108 ± 12
GC	10	62.1 ± 3.5
TC	10	72.4 ± 9.8
GLC	10	15.9 ± 0.6
DHEAS	10	196 ± 47
Sildenafil	10	78.9 ± 2.5
MK571	1	82.3 ± 4.7
MK571	10	25.0 ± 3.4
Probenecid	10	75.6 ± 5.9
Indomethacin	10	64.1 ± 10
Sulfanitran	10	129 ± 3.8
Sulfinpyrazone	10	146 ± 3.5
Penicillin G	10	91.1 ± 1.9

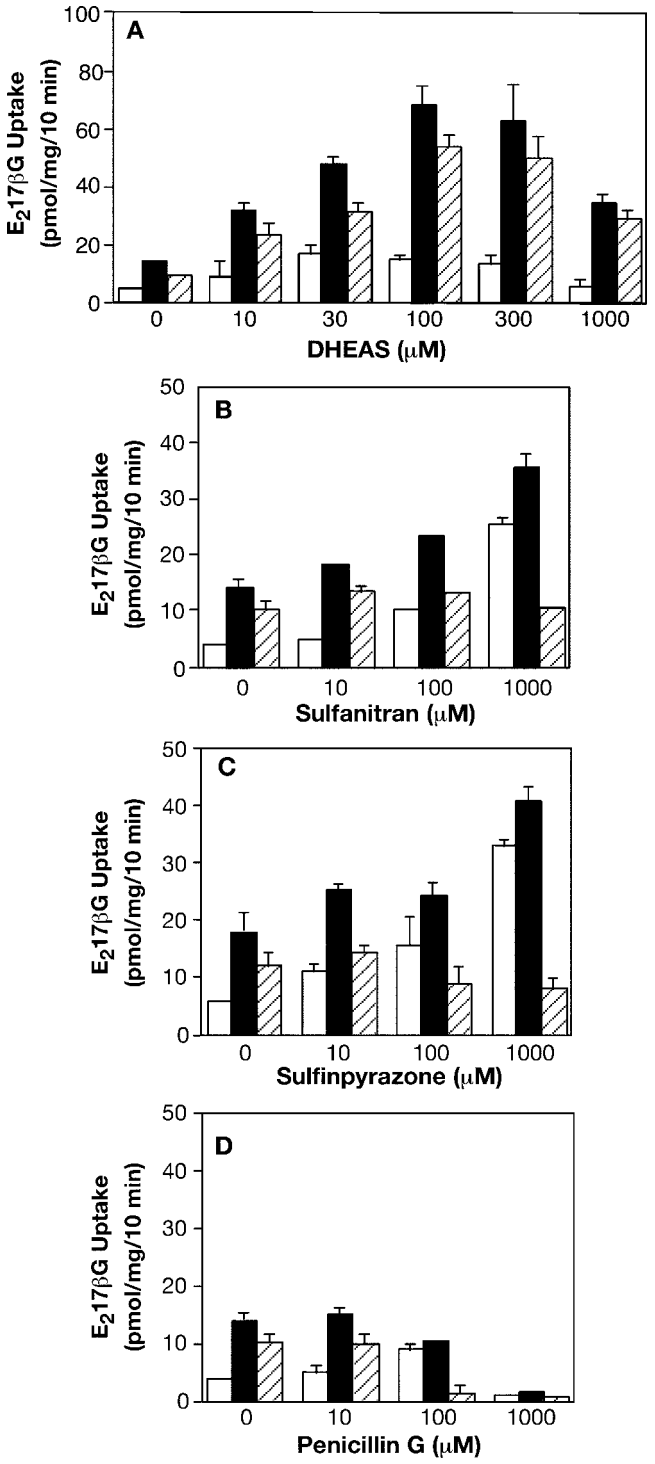

Fig. 11. Effects of DHEAS, sulfanitran, sulfinpyrazone, and penicillin G on MRP8-mediated transport. Membrane vesicles prepared from LLC-PK1-MRP8-3 or LLC-PK1-pcDNA were incubated at 37°C for 10 min in uptake medium containing 1 μM [^{3}H]$E_2$17βG in the presence of varying concentrations of DHEAS (A), sulfanitran (B), sulfinpyrazone (C), or penicillin G (D). White columns, ATP-dependent uptake by LLC-PK1-pcDNA membrane vesicles; black columns, ATP-dependent uptake by LLC-PK1-pcDNA-8-3 membrane vesicles; striped columns, MRP8-dependent uptake. ATP-dependent uptake was enumerated as uptake in medium containing 4 mM MgATP minus uptake by the same membrane vesicles in medium containing 4 mM MgAMP. MRP8-dependent uptake was enumerated as ATP-dependent uptake by MRP8-enriched membrane vesicles minus ATP-dependent uptake by control membrane vesicles. The values shown are means ± S.E. of a representative experiment performed in duplicate.

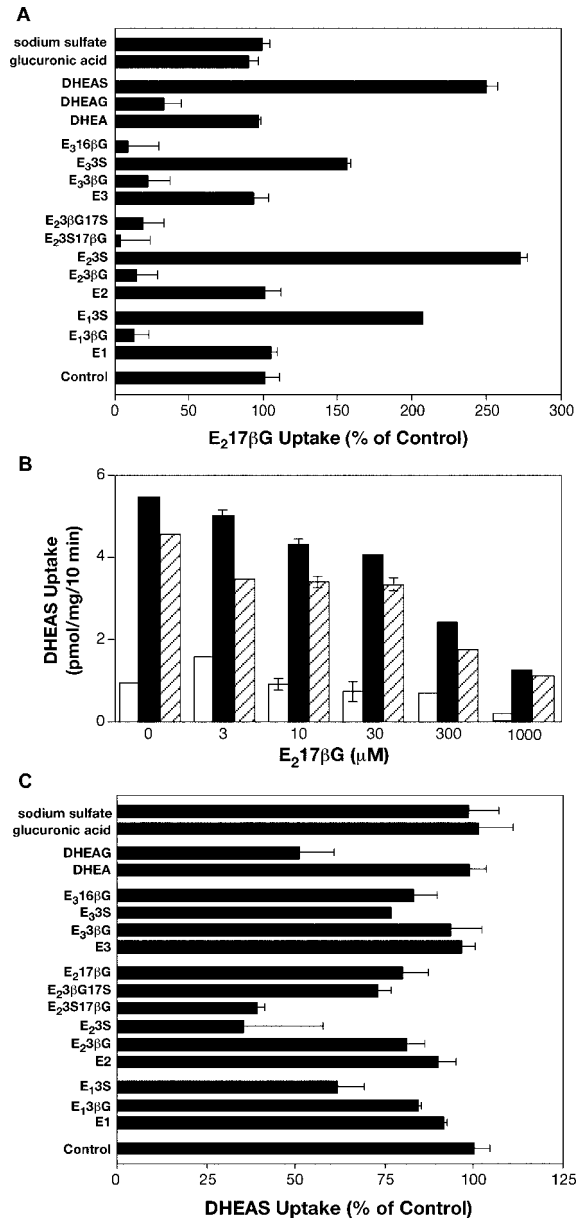

Fig. 12. Effects modified and unmodified steroids on $E_2$17βG and DHEAS uptake. A, effects of modified and unmodified steroids on $E_2$17βG uptake. Membrane vesicles prepared from LLC–PK1-MRP8-3 were incubated at 37°C for 10 min in uptake medium containing 1 μM [^{3}H]$E_2$17βG in the presence of 10 μM concentrations of the indicated compounds. ATP-dependent uptake was enumerated as uptake in medium containing 4 mM MgATP minus uptake by the same membrane vesicles in medium containing 4 mM MgAMP. The values shown are means ± S.E. of three experiments performed in duplicate. B, effects of varying concentrations of $E_2$17βG on DHEAS uptake. Membrane vesicles prepared from LLC-PK1-MRP8-3 or LLC-PK1-pcDNA were incubated at 37°C for 10 min in uptake medium containing 100 nM [^{3}H]DHEAS in the presence of varying concentrations of $E_2$17βG. White columns, ATP-dependent uptake by LLC-PK1-pcDNA membrane vesicles; black columns, ATP-dependent uptake by LLC-PK1-MRP8-3 membrane vesicles; striped columns, MRP8-dependent uptake. ATP-dependent uptake was enumerated as uptake in medium containing 4 mM MgATP minus uptake by the same membrane vesicles in medium containing 4 mM MgAMP. MRP8-dependent uptake was enumerated as ATP-dependent uptake by MRP8-enriched membrane vesicles minus ATP-dependent uptake by control membrane vesicles. The values shown are means ± S.E. of a representative experiment performed in duplicate. C, effects of various modified and unmodified steroids on DHEAS uptake. Membrane vesicles prepared from LLC-PK1-MRP8-3 were incubated at 37°C for 10 min in uptake medium containing 100 nM [^{3}H]DHEAS in the presence of 10 μM concentrations of the indicated compounds. The values shown are means ± S.E. of ATP-dependent uptake for three experiments performed in duplicate.

(19.8% at 1 μM), DNP-SG (43.7% at 10 μM), glycolithocholate (71% at 10 μM), and MK571 (78.4% at 10 μM) were the most potent inhibitors of DHEAS transport, and MTX (22.5%) was a more potent inhibitor compared with lower polyglutamyl MTX species (~4%).

The effects of conjugated steroids on DHEAS transport were examined in more detail. The inhibition exerted by $E_2$17βG on MRP8-dependent transport was concentration-dependent, increasing from 23.9% at 3 μM to 75% at 300 μM (Fig. 12B). Of the other conjugated steroids analyzed, in no case was stimulation of DHEAS uptake observed (Fig. 12C). DHEAG was the most potent inhibitor of the steroids bearing only glucuronate substituents (49.1%). The estrogen glucuronides, irrespective of whether the glucuronic acid modification is present on the D ring ($E_3$16βG, 16.8%; $E_2$17βG, 19.9%), or the A ring ($E_1$3βG, 15.7%; $E_2$3βG, 18.8%; $E_3$3βG, 6.6%), exerted a modest increment in inhibition over that observed for the respective unmodified parent compounds. The 3′ sulfated estrogens were more potent inhibitors than the corresponding 3′ estrogen glucuronides, exerting inhibitions of 38.5% ($E_1$3S), 64.8% ($E_2$3S), and 23.2% ($E_3$3S). The inhibition exerted by $E_2$3S17βG (61.1%) was comparable with that of $E_2$3S, and the inhibition exerted by $E_2$3βG17S (26.9%) was only modestly greater than $E_2$3βG. Neither glucuronic acid nor sodium sulfate exerted appreciable inhibitions, at either 10 μM (Fig. 12C) or 1 mM (data not shown).

Effects of DHEAS and $E_2$17βG on Taurocholate Transport. Having determined the effects of DHEAS and $E_2$17βG on transport of each other, and the effects of TC on transport of DHEAS and $E_2$17βG (Tables 2 and 3), we next analyzed the effects of DHEAS and $E_2$17βG on TC transport. As shown in Fig. 13, each of these compounds exerted a dose-dependent inhibition of TC uptake.

TABLE 3

Effects of various compounds on [^{3}H]DHEAS transport

Membrane vesicles prepared from LLC/PK1-MRP8-3 cells were incubated at 37° C for 10 min in media containing 100 nM [^{3}H]DHEAS in the presence of the indicated compounds. ATP-dependent uptake was calculated by subtracting values obtained in the presence of 4 mM ATP from those in the presence of 4 mM AMP. Transport is expressed as percentage uptake in the absence of modulator. Values shown are means ± S.E. of at least three measurements performed in duplicate.

Compound	Concentration	Uptake
	μM	*% of control*
Control		100
cAMP	10	86.2 ± 3.7
cGMP	10	82.3 ± 6.7
LTC_4	1	80.2 ± 5.3
DNP-SG	1	92.4 ± 6.8
DNP-SG	10	56.3 ± 8.9
MTX	10	77.5 ± 11
MTX-Glu2	10	96.4 ± 5.3
MTX-Glu3	10	95.5 ± 10
GC	10	65.7 ± 4.6
TC	10	66.6 ± 1.7
GLC	10	29.0 ± 14
$E_2$17βG	10	80.3 ± 11
Sildenafil	10	82.4 ± 5.4
MK571	1	85.6 ± 9.3
MK571	10	21.6 ± 15
Probenecid	10	91.4 ± 8.9
Indomethacin	10	76.4 ± 2.2
Sulfanitran	10	101 ± 10
Sulfinpyrazone	10	92.1 ± 5.6
Penicillin G	10	98.3 ± 7.0

Discussion

In the present study, the in vitro transport characteristics of MRP8 were investigated to gain insights into the substrate range, potential in vivo functions, and biochemical activity of the pump. The results of these experiments provide the first direct determination of the substrates of MRP8. The membrane vesicle uptake experiments reveal that a range of physiological amphipathic anions, as exemplified by LTC_4, $E_2$17βG, DHEAS, E1S, GC, and TC are MRP8 substrates. In addition, it is shown that MRP8 is capable of transporting cAMP and cGMP, a finding that is in accord with our previous cellular experiments showing that expression of MRP8 depresses intracellular levels of cyclic nucleotides by enhancing cellular extrusion, either under basal conditions or when cells were treated with agents that stimulate the synthesis of cyclic nucleotides (Guo et al., 2003). Hence, MRP8 is able to transport a variety of lipophiles conjugated to glutathione, glucuronate, sulfate, glycine, and taurine, as well as to phosphate.

Although amino acid comparisons indicate that MRP8 more closely resembles MRP5 than it does MRP4 (Bera et al., 2001; Tammur et al., 2001; Yabuuchi et al., 2001), our results indicate that the substrate selectivity of MRP8 is more similar to that of MRP4. Both of these pumps are able to transport glucuronate and glutathione conjugates, monoanionic bile acids, DHEAS, E1S, and folates (Chen et al., 2001, 2002; Zelcer et al., 2003b). However, there are distinguishing features in the selectivities of the two pumps. Transport of GC and TC by MRP4 exhibits a strict glutathione dependence, and MRP4 is able to transport prostaglandins such as PGE1 and PGE2 (Reid et al., 2003; Rius et al., 2003). By comparison, we found that MRP8 is able to transport monoanionic bile acids in the absence of glutathione, and that MRP8-mediated transport of PGE1 and PGE2 was undetectable under the experimental conditions used in our uptake assays. At the time of writing, cyclic nucleotides are the only physiological transport substrates that MRP4, MRP5, and MRP8 are known to have in common (Jedlitschky et al., 2000; Chen et al., 2001; van Aubel et al., 2002; Guo et al., 2003). However, all three

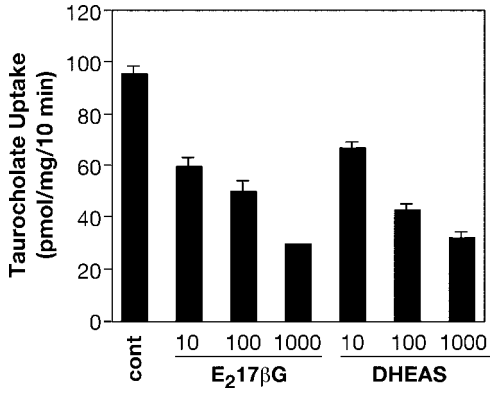

Fig. 13. Effects of $E_2$17βG and DHEAS on taurocholate transport. Membrane vesicles prepared from LLC-PK1-MRP8-3 were incubated at 37°C for 10 min in uptake medium containing 10 μM[^{3}H]TC in the presence of varying concentrations of $E_2$17βG or DHEAS. ATP-dependent uptake was enumerated as uptake in medium containing 4 mM MgATP minus uptake by the same membrane vesicles in medium containing 4 mM MgAMP. The values shown are means ± S.E. of two experiments performed in duplicate.

pumps are able to confer resistance to nucleotide analogs such as 9-(2-phosphonylmethoxyethyl)adenine (Schuetz et al., 1999; Lee et al., 2000; Wijnholds et al., 2000; Chen et al., 2001; Guo et al., 2003).

A striking finding to come from these investigations, and another feature that distinguishes the biochemical activity of MRP8 from MRP4, is the susceptibility of MRP8-mediated transport of $E_2$17βG to stimulation by DHEAS. By contrast, sulfated steroids were reported to inhibit $E_2$17βG transport by MRP4, and DHEAS was a particularly potent inhibitor (Zelcer et al., 2003b). Two potential mechanisms that could account for constructive interactions between some transport substrates of MRP8 are that $E_2$17βG and DHEAS are cotransported from a single substrate binding site or that DHEAS stimulates transport of $E_2$17βG by binding to a topologically distinct site. Two findings tend to support the latter possibility. First, stimulation of $E_2$17βG transport was attenuated at high DHEAS concentrations, a finding that is reminiscent of the activating effects of compounds such as indomethacin on MRP2-mediated transport of $E_2$17βG (Bodo et al., 2003; Zelcer et al., 2003a) and suggests that at lower concentrations DHEAS exerts a stimulatory effect by binding to a site distinct from the transport substrate binding pocket, whereas at higher concentrations it may predominately compete with $E_2$17βG binding at the transport site. Second, the interaction between $E_2$17βG and DHEAS is not reciprocal, as might be expected for cotransport, in that instead of promoting DHEAS transport $E_2$17βG exerted an inhibitory effect. This finding, in combination with the results showing that several estrogens bearing 3′ sulfate substituents seemed to stimulate $E_2$17βG transport, whereas the corresponding 3′ glucuronidated compounds and DHEAG inhibited transport, and that MRP8-mediated transport of TC is susceptible to inhibition by both $E_2$17βG and DHEAS, indicate that the putative second binding site invoked above, as well as the transported substrate, are specific in terms of the ability of compounds to stimulate transport in the former case, and the susceptibility of transport substrates to stimulation in the latter case. The invocation of a mechanism that does not involve cotransport to explain the stimulatory effect of DHEAS on MRP8-mediated transport is further supported by the recent finding that ethinylestradiol sulfate, which is not a transport substrate of MRP2 or MRP3, is able to stimulate $E_2$17βG transport by both of these pumps (Chu et al., 2004). A potential kinetic manifestation of separate binding sites in MRP8 for sulfated steroids—a sigmoidal concentration dependence curve for DHEAS transport—could not be evaluated in our study because of appreciable uptake rates by the control membranes. Additional transport and protein structural studies are needed to further define the cooperative interactions between some MRP8 substrates.

MRP8 transcript has been reported to be expressed in a wide range of normal tissues, including liver, breast, prostate, and testis (Bera et al., 2001; Tammur et al., 2001; Yabuuchi et al., 2001). This expression pattern, in combination with the determination of MRP8 substrates described herein, allows for speculation as to potential physiological functions of the pump. The ability of MRP8 to mediate the transport of monoanionic bile acids is of particular interest in view of MRP8 transcript expression in liver, because this suggests that the pump may be involved in bile acid homeostasis in hepatocytes. Two other MRP family members that are competent in bile acid transport, MRP3 and MRP4, have previously been implicated in this process. MRP3, which is induced at basolateral surfaces of hepatocytes in cholestatic conditions, has been proposed as a backup system that functions to extrude bile acids and conjugates such as bilirubin glucuronide into portal blood when the normal canalicular route mediated by bile salt export pump (bile acids) and MRP2 (conjugates) is impaired (Konig et al., 1999; Hirohashi et al., 2000; Zeng et al., 2000). This physiological role is supported by experiments showing that *Mrp3* null mice, when made cholestatic by bile duct ligation, have increased hepatic bile acid levels and reduced serum levels of bilirubin glucuronide (M. G. Belinsky and G. D. Kruh, unpublished data). The finding that MRP4, which also assumes basolateral localization in hepatocytes, is up-regulated in cholestatic conditions indicates that it may play a similar role (Rius et al., 2003). The facility of MRP8 for transporting glucuronidated and sulfated steroids such as $E_2$17βG, DHEAS, and $E_1$3S, in combination with MRP8 transcript expression in breast, prostate, and testis, suggests that the pump may play a role in the response of these hormonally regulated tissues to sex steroids. Along the same lines, the reported expression of MRP8 transcript in breast cancer specimens is noteworthy in that the hormone responsiveness of this cancer is highly relevant to its treatment (Bera et al., 2001). Additional insights into the physiological functions of the pump, and its relevance to cancer, should be afforded by investigations of MRP8 protein expression in normal tissues and cancers, and by determination of its subcellular localization in polarized epithelial cells.

The determination that MRP8 is able to transport MTX extends our understanding of the molecular components of the energy-dependent membrane system that mediates extrusion of this prototypical antifolate. Because the retention, and associated cytotoxicity of MTX, is dependent upon the ability of cells to transport MTX, but not the polyglutamyl species to which MTX is rapidly converted intracellularly, the molecular identification of components of the efflux system has been of substantial interest (Zhao and Goldman, 2003). In previous studies we characterized the in vitro transport capabilities of several MRP family members toward MTX, and found that MRP1, MRP2, MRP3, and MRP4, but not MRP6 or MRP7, are competent in the transport of this agent (Zeng et al., 2000, 2001; Belinsky et al., 2002; Chen et al., 2002, 2003a). As would be expected for components of the MTX efflux system, we determined that the robust activity of MRPs 1 to 4 toward MTX is severely attenuated by the addition of even one more glutamyl residue and that these pumps are high-capacity, low-affinity transporters of unmodified MTX (Zeng et al., 2001; Chen et al., 2002). ABCG2 is also capable of mediating the high-capacity, low-affinity transport of MTX (Chen et al., 2003b; Volk and Schneider, 2003). However, by contrast with MRPs 1 to 4, ABCG2 is also able to transport diglutamyl and triglutamyl MTX species. A consequence of the MTX transport properties exhibited by MRPs 1 to 4 is that these pumps are potent resistance factors when transfected cells are exposed to high drug concentrations for the first few hours of a 3-day growth

assay, but weak resistance factors in continuous exposure assays in which much lower MTX concentrations are used (Hooijberg et al., 1999; Kool et al., 1999; Lee et al., 2000). The results showing that MRP8 is a high-capacity, low-affinity transporter (K_m = 957 μM) of this agent and that it has little or no activity toward MTX polyglutamates indicate that the MTX transport characteristics of MRP8 are similar to those of MRPs 1 to 4 and that the plasma membrane efflux system responsible for MTX extrusion is composed of no less than six ATP-binding cassette transporters. We anticipate that MRP8 will also have the facility for conferring resistance to this agent. In that case, the resistance profile of MRP8 would encompass two of three components of a chemotherapeutic regimen (cytoxan, MTX, and 5-fluorouracil) used in the adjuvant treatment of breast cancer. This circumstance, in combination with the considerations mentioned above, would further suggest that investigation of MRP8 expression in this cancer is warranted.

Acknowledgments

We thank Philip Rea (University of Pennsylvania) for critically reviewing the manuscript.

References

Akita H, Suzuki H, Hirohashi T, Takikawa H, and Sugiyama Y (2002) Transport activity of human MRP3 expressed in Sf9 cells: comparative studies with rat MRP3. *Pharm Res (NY)* **19:**34–41.

Awasthi YC, Garg HS, Dao DD, Partridge CA, and Srivastava SK (1981) Enzymatic conjugation of erythrocyte glutathione with 1-chloro-2,4-dinitrobenzene: the fate of glutathione conjugate in erythrocytes and the effect of glutathione depletion on hemoglobin. *Blood* **58:**733–738.

Belinsky MG, Bain LJ, Balsara BB, Testa JR, and Kruh GD (1998) Characterization of MOAT-C and MOAT-D, new members of the MRP/cMOAT subfamily of transporter proteins. *J Natl Cancer Inst* **90:**1735–1741.

Belinsky MG, Chen ZS, Shchaveleva I, Zeng H, and Kruh GD (2002) Characterization of the drug resistance and transport properties of multidrug resistance protein 6 (MRP6, ABCC6). *Cancer Res* **62:**6172–6177.

Bera TK, Lee S, Salvatore G, Lee B, and Pastan IH (2001) MRP8, a new member of ABC transporter superfamily, identified by EST database mining and gene prediction program, is highly expressed in breast cancer. *Mol Med* **7:**509–516.

Bodo A, Bakos E, Szeri F, Varadi A, and Sarkadi B (2003) Differential modulation of the human liver conjugate transporters MRP2 and MRP3 by bile acids and organic anions. *J Biol Chem* **278:**23529–23537.

Bradford HF and Ward HK (1976) On glutaminase activity in mammalian synaptosomes. *Brain Res* **110:**115–125.

Chen Z-S, Hopper-Borge E, Belinsky MG, Shchaveleva I, Kotova E, and Kruh GD (2003a) Characterization of the transport properties of human multidrug resistance protein 7 (MRP7, ABCC10). *Mol Pharmacol* **63:**351–358.

Chen Z-S, Lee K, and Kruh GD (2001) Transport of cyclic nucleotides and estradiol 17-β-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine. *J Biol Chem* **276:**33747–33754.

Chen Z-S, Lee K, Walther S, Blanchard Raftogianis R, Kuwano M, Zeng H, and Kruh GD (2002) Analysis of methotrexate and folate transport by multidrug resistance protein 4: MRP4 is a component of the methotrexate efflux system. *Cancer Res* **62:**3144–3150.

Chen Z-S, Robey RW, Belinsky MG, Shchaveleva I, Ren XQ, Sugimoto Y, Ross DD, Bates SE, and Kruh GD (2003b) Transport of methotrexate, methotexate polyglutamates and 17β-estradiol 17-(β-D-glucuronide) by ABCG2 (BCRP, MXR): Effects of acquired mutations at amino acid position 482 and polyglutamylation on methotrexate transport. *Cancer Res* **63:**4048–4054.

Chu XY, Huskey SE, Braun MP, Sarkadi B, Evans DC, and Evers R (2004) Transport of ethinylestradiol glucuronide and ethinylestradiol sulfate by the multidrug resistance proteins MRP1, MRP2 and MRP3. *J Pharmacol Exp Ther* **309:**156–164.

Cornwell MM, Gottesman MM, and Pastan IH (1986) Increased vinblastine binding to membrane vesicles from multidrug-resistant KB cells. *J Biol Chem* **261:**7921–7928.

Cui Y, Konig J, Buchholz JK, Spring H, Leier I, and Keppler D (1999) Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. *Mol Pharmacol* **55:**929–937.

Gerk PM and Vore M (2002) Regulation of expression of the multidrug resistance-associated protein 2 (MRP2) and its role in drug disposition. *J Pharmacol Exp Ther* **302:**407–415.

Guo Y, Kotova E, Chen Z-S, Lee K, Hopper-Borge E, Belinsky MG, and Kruh GD (2003) MRP8, ATP-binding cassette C11 (ABCC11), is a cyclic nucleotide efflux pump and a resistance factor for fluoropyrimidines, 2′,3′-dideoxycytidine and 9′-(2′-phosphonylmethoxyethyl)adenine. *J Biol Chem* **278:**29509–29514.

Haimeur A, Conseil G, Deeley RG, and Cole SP (2004) The MRP-related and BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and regulation. *Curr Drug Metab* **5:**21–53.

Hirohashi T, Suzuki H, and Sugiyama Y (1999) Characterization of the transport properties of cloned rat multidrug resistance-associated protein 3 (MRP3). *J Biol Chem* **274:**15181–15185.

Hirohashi T, Suzuki H, Takikawa H, and Sugiyama Y (2000) ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3). *J Biol Chem* **275:**2905–2910.

Hooijberg JH, Broxterman HJ, Kool M, Assaraf YG, Peters GJ, Noordhuis P, Scheper RJ, Borst P, Pinedo HM, and Jansen G (1999) Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. *Cancer Res* **59:**2532–2535.

Ilias A, Urban Z, Seidl TL, Le Saux O, Sinko E, Boyd CD, Sarkadi B, and Varadi A (2002) Loss of ATP-dependent transport activity in pseudoxanthoma elasticum-associated mutants of human ABCC6(MRP6). *J Biol Chem* **277:**16860–16867.

Ito K, Suzuki H, Hirohashi T, Kume K, Shimizu T, and Sugiyama Y (1998) Functional analysis of a canalicular multispecific organic anion transporter cloned from rat liver. *J Biol Chem* **273:**1684–1688.

Jedlitschky G, Burchell B, and Keppler D (2000) The multidrug resistance protein 5 functions as an ATP-dependent export pump for cyclic nucleotides. *J Biol Chem* **275:**30069–30074.

Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G, and Keppler D (1996) Transport of glutathione, glucuronate and sulfate conjugates by the MRP gene-encoded conjugate export pump. *Cancer Res* **56:**988–994.

Kawabe T, Chen Z-S, Wada M, Uchiumi T, Ono M, Akiyama S, and Kuwano M (1999) Enhanced transport of anticancer agents and leukotriene C4 by the human canalicular multispecific organic anion transporter (cMOAT/MRP2). *FEBS Lett* **456:**327–331.

Konig J, Rost D, Cui Y, and Keppler D (1999) Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. *Hepatology* **29:**1156–1163.

Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, et al. (1999) MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc Natl Acad Sci USA* **96:**6914–6919.

Kruh GD and Belinsky MG (2003) The MRP family of drug efflux pumps. *Oncogene* **22:**7537–7552.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* **227:**680–685.

Lee K, Klein-Szanto AJ, and Kruh GD (2000) Analysis of the MRP4 drug resistance profile in transfected NIH3T3 cells. *J Natl Cancer Inst* **92:**1934–1940.

Leier I, Jedlitschky G, Buchholz U, Cole SP, Deeley RG, and Keppler D (1994) The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. *J Biol Chem* **269:**27807–27810.

Leslie EM, Ito K, Upadhyaya P, Hecht SS, Deeley RG, and Cole SP (2001) Transport of the beta-O-glucuronide conjugate of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by the multidrug resistance protein 1 (MRP1). Requirement for glutathione or a non-sulfur-containing analog. *J Biol Chem* **276:**27846–27854.

Liu G, Sanchez-Fernandez R, Li ZS, and Rea PA (2001) Enhanced multispecificity of *Arabidopsis* vacuolar multidrug resistance-associated protein-type ATP-binding cassette transporter, AtMRP2. *J Biol Chem* **276:**8648–8656.

Loe DW, Almquist KC, Cole SP, and Deeley RG (1996) ATP-dependent 17β-estradiol 17-(β-D-glucuronide) transport by multidrug resistance protein (MRP). Inhibition by cholestatic steroids. *J Biol Chem* **271:**9683–9689.

Madon J, Hagenbuch B, Landmann L, Meier PJ, and Stieger B (2000) Transport function and hepatocellular localization of mrp6 in rat liver. *Mol Pharmacol* **57:**634–641.

Marquardt DW (1963) An algorithm for nonlinear estimation of nonlinear parameters. *J Soc Ind Appl Math* **11:**431–441.

Qian YM, Song WC, Cui H, Cole SP, and Deeley RG (2001) Glutathione stimulates sulfated estrogen transport by multidrug resistance protein 1. *J Biol Chem* **276:**6404–6411.

Reid G, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, Wijnholds J, and Borst P (2003) The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proc Natl Acad Sci USA* **100:**9244–9249.

Rius M, Nies AT, Hummel-Eisenbeiss J, Jedlitschky G, and Keppler D (2003) Cotransport of reduced glutathione with bile salts by MRP4 (ABCC4) localized to the basolateral hepatocyte membrane. *Hepatology* **38:**374–384.

Schuetz JD, Connelly MC, Sun D, Paibir SG, Flynn PM, Srinivas RV, Kumar A, and Fridland A (1999) MRP4: A previously unidentified factor in resistance to nucleoside-based antiviral drugs. *Nat Med* **5:**1048–1051.

Tammur J, Prades C, Arnould I, Rzhetsky A, Hutchinson A, Adachi M, Schuetz JD, Swoboda KJ, Ptacek LJ, Rosier M, et al. (2001) Two new genes from the human ATP-binding cassette transporter superfamily, ABCC11 and ABCC12, tandemly duplicated on chromosome 16q12. *Gene* **273:**89–96.

Towbin H, Staehelin T, and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76:**4350–4354.

van Aubel RA, Smeets PH, Peters JG, Bindels RJ, and Russel FG (2002) The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. *J Am Soc Nephrol* **13:**595–603.

Volk EL and Schneider E (2003) Wild-type breast cancer resistance protein (BCRP/ABCG2) is a methotrexate polyglutamate transporter. *Cancer Res* **63:**5538–5543.

Wielinga PR, Van Der Heijden I, Reid G, Beijnen JH, Wijnholds J, and Borst P (2003) Characterization of the MRP4-and MRP5-mediated transport of cyclic nucleotides from intact cells. *J Biol Chem* **278:**17664–17671.

Wijnholds J, Mol CA, van Deemter L, de Haas M, Scheffer GL, Baas F, Beijnen JH, Scheper RJ, Hatse S, De Clercq E, et al. (2000) Multidrug-resistance protein 5 is

a multispecific organic anion transporter able to transport nucleotide analogs. *Proc Natl Acad Sci USA* **97:**7476–7481.
Yabuuchi H, Shimizu H, Takayanagi S, and Ishikawa T (2001) Multiple splicing variants of two new human ATP-binding cassette transporters, ABCC11 and ABCC12. *Biochem Biophys Res Commun* **288:**933–939.
Zelcer N, Huisman MT, Reid G, Wielinga P, Breedveld P, Kuil A, Knipscheer P, Schellens JH, Schinkel AH, and Borst P (2003a) Evidence for two interacting ligand binding sites in human multidrug resistance protein 2 (ATP binding cassette C2). *J Biol Chem* **278:**23538–23544.
Zelcer N, Reid G, Wielinga P, Kuil A, Van Der Heijden I, Schuetz JD, and Borst P (2003b) Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4). *Biochem J* **371:**361–367.
Zeng H, Chen Z-S, Belinsky MG, Rea PA, and Kruh GD (2001) Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport. *Cancer Res* **61:**7225–7232.
Zeng H, Liu G, Rea PA, and Kruh GD (2000) Transport of amphipathic anions by human multidrug resistance protein 3. *Cancer Res* **60:**4779–4784.
Zhao R and Goldman ID (2003) Resistance to antifolates. *Oncogene* **22:**7431–7457.

Address correspondence to: Dr. Gary D. Kruh, Fox Chase Cancer Center, 333 Cottman Ave., Philadelphia, PA 19111. E-mail: gd_kruh@fccc.edu.