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Transport of Glutathione, Glucuronate, and Sulfate Conjugates by the *MRP* Gene-encoded Conjugate Export Pump[1]

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ABSTRACT

Previous studies have identified the ATP-dependent export of glutathione conjugates as a physiological function of the multidrug resistance protein (MRP). The involvement of MRP in the transport of endogenous and xenobiotic conjugates was investigated further using membrane vesicles from *MRP*-transfected HeLa cells. The ATP-dependent transport of the glutathione conjugates [^{3}H]leukotriene C_4, *S*-(2,4-dinitrophenyl)-[^{3}H]glutathione, and ^{3}H-labeled oxidized glutathione was characterized by determination of the transport efficiency V_{max}:K_m amounting to 1031, 114, and 7.1 ml × mg protein^{-1} × min^{-1}, respectively. Additional endogenous substrates for MRP-mediated transport included the steroid conjugate 17β-glucuronosyl [^{3}H]estradiol and the bile salt conjugates [6α-^{14}C]glucuronosylhyodeoxycholate and 3α-sulfatolithocholyl [^{3}H]taurine. The K_m value of MRP for 17β-glucuronosyl [^{3}H]estradiol was 1.5 ± 0.3 μM, with a V_{max}:K_m ratio of 42 ml × mg protein^{-1} × min^{-1}, and a K_i value of 0.7 μM for the leukotriene receptor antagonist MK 571. MRP-mediated ATP-dependent transport was observed for the anticancer drug conjugates glucuronosyl [^{3}H]etoposide and monochloro-mono[^{3}H]glutathionyl melphalan, but not for unmodified [^{14}C]doxorubicin, [^{3}H]daunorubicin, or [^{3}H]vinblastine. Our results establish that MRP functions as an ATP-dependent export pump not only for glutathione conjugates but also for glucuronidated and sulfated endogenous as well as exogenous compounds.

INTRODUCTION

The MRP[3] (also multidrug resistance-associated protein) has been identified as a member of the ATP-binding cassette transporter superfamily (1, 2) and was shown to function in the ATP-dependent transport of the endogenous glutathione *S*-conjugate LTC_4 (3–5). MRP was discovered because of its overexpression in a number of multidrug-resistant human tumor cell lines that do not overexpress the MDR1 P-glycoprotein (1, 2, 6–8). In addition, MRP was shown to be expressed in several normal tissues (1, 8) including the liver (9). ATP-dependent transport of LTC_4 in liver has been localized to the canalicular membrane domain (10), and this transport process is deficient in a mutant strain of rats (GY/TR$^-$) (10, 11). These mutants have been valuable in defining the substrate specificity of this export pump in the rat, which was termed multispecific organic anion transporter (12), nonbile acid organic anion transporter (13), or glutathione *S*-conjugate export pump (14). Comparison of compounds secreted into bile of normal and GY/TR$^-$ rats indicates that a wide range of glutathione, glucuronate, and sulfate conjugates, as well as additional amphiphilic anions, may be transported by the same system (11, 12, 15, 16). Our recent finding of the selective absence of an isoform of Mrp (rat multidrug resistance protein) from the canalicular membrane of GY/TR$^-$ mutant rat liver indicates that this canalicular Mrp isoform mediates the hepatobiliary excretion of substrates not present in the bile of these mutants (9).

The availability of *MRP*-transfected and control HeLa cells (17) enabled us in the present study to define substrates of the human *MRP*-encoded export pump by direct measurements of ATP-dependent transport into inside-out-oriented membrane vesicles prepared from these MRP-overexpressing and control cells. Compounds tested for transport include endogenous glucuronate and sulfate conjugates of steroids as well as glucuronate- and glutathione-conjugated anticancer drugs, in addition to the formerly identified glutathione-derived substrates. These studies identify MRP as an ATP-dependent export pump with a broad specificity for amphiphilic anions, most of which are conjugates of lipophilic compounds with glutathione, glucuronate, or sulfate.

MATERIALS AND METHODS

Materials. [14,15,19,20-^{3}H]LTC_4 (46.4 TBq/mmol), [14,15,19,20-^{3}H]LTD_4 (6.4 TBq/mmol), [14,15,19,20-^{3}H]LTE_4 (6.4 TBq/mmol), [5,6,8,9,11,12,14,15-^{3}H]LTB_4 (7.4 TBq/mmol), [5,6,8,9,11,12,14,15-^{3}H]thromboxane B_2 (2.3 TBq/mmol), [5,6,8,9,11,12,14,15-^{3}H]prostaglandin D_2 (4.3 TBq/mmol), [^{3}H]taurocholic acid ([^{3}H]cholyltaurine; 74 GBq/mmol), [6,7-^{3}H]estradiol (1.6 TBq/mmol), [6,7-^{3}H]estradiol 17β-D-glucuronide (1.8 TBq/mmol), [^{3}H]daunorubicin ([^{3}H]daunomycin; 0.2 TBq/mmol), and [glycine-2-^{3}H]glutathione ([^{3}H]GSH; 1.6 TBq/mmol) were obtained from DuPont-New England Nuclear (Boston, MA). *N*-acetylation of [14,15,19,20-^{3}H]LTE_4 and LTE_4 was performed with acetic anhydride (18). UDP [^{14}C]glucuronic acid (10.6 GBq/mmol) was obtained from ICN Biomedicals, Inc. (Irvine, CA). [Chloroethyl-1,2-^{14}C]melphalan (1.9 GBq/mmol) and [^{3}H]etoposide (37 GBq/mmol) were obtained from Moravek Biochemicals, Inc. (Brea, CA). [14-^{14}C]Doxorubicin ([14-^{14}C]Adriamycin; 2.1 GBq/mmol), [^{3}H]vinblastine (0.4 TBq/mol), and unlabeled LTC_4 were obtained from Amersham Buchler (Braunschweig, Germany). Unlabeled GSH, GSSG, hyodeoxycholate, sulfatolithocholyltaurine, 3′-phosphoadenosine 5′-phosphosulfate, UDPGlcA, UDPGlcA β-D-glucuronosyltransferase (EC 2.4.1.17) from bovine liver, glutathione *S*-transferase (EC 2.5.1.18) from rat liver, melphalan [L-*p*-(di-2-chloro-ethylamino)phenylalanine mustard], and etoposide were obtained from Sigma Chemical Co. (St. Louis, MO). β-Glucuronidase/arylsulfatase (EC 3.2.1.31/EC 3.1.6.1) was obtained from Boehringer Mannheim (Mannheim, Germany).The LTD_4 receptor antagonist MK 571, which is a quinoline-based amphiphilic anion (19), was kindly provided by Dr. A. W. Ford-Hutchinson (Merck-Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Montreal, Quebec, Canada). Nitrocellulose filters (pore size 0.2 μm) were obtained from Schleicher & Schüll (Dassel, Germany). Nick spin columns filled with Sephadex G-50 fine were purchased from Pharmacia-LKB (Freiburg, Germany).

Synthesis of Glutathione *S*-Conjugates. ^{3}H-labeled glutathione conjugates and [^{3}H]GSSG were synthesized from [^{3}H]GSH after removal of the DTT from the commercially delivered solution by ethyl acetate extraction. [^{3}H]GSSG was synthesized nonenzymatically in 10 mM Tris/HCl buffer (pH 7.4) containing 1 mM H_2O_2 (20). DNP-[^{3}H]SG and unlabeled DNP-SG were prepared from [^{3}H]GSH or GSH and 1-chloro-2,4-dinitrobenzene using glutathione *S*-transferase from rat liver (20). The purity of [^{3}H]GSSG was tested by HPLC separation using a NH_2-Spherisorb column and a water/methanol/acetic acid gradient for elution. For DNP-[^{3}H]SG purification, a C_{18} Hypersil column and a water/acetonitrile gradient were used.

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[3] The abbreviations used are: MRP, multidrug resistance protein; LT, leukotriene; AMP-PCP, adenosine 5′-[β,γ-methylene]-triphosphate; DNP-SG, *S*-(2 4-dinitrophenyl)-glutathione; ESI-MS/MS, electrospray ionization mass spectrometry; GSH, reduced glutathione; GSSG, oxidized glutathione or glutathione disulfide; HPLC, high-performance liquid chromatography; 6α-glucuronosylhyodeoxycholate, 6α-glucuronosyl-3-hydroxy-5β-cholan-24-oate; LTE_4NAc, *N*-acetyl-LTE_4; melphalan, L-phenylalanine mustard; MK 571, 3-({[3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl]-{(3-dimethylamino-3-oxopropyl)-thio}-methyl]thio)propanoic acid; sulfatolithocholyltaurine, 3α-sulfato-5β-cholan-24-oyl)-2′-aminoethanesulfonate; UDPGlcA, UDP glucuronate.

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Melphalan was conjugated with [^{3}H]GSH by incubation with cytosol (100,000 × *g* supernatant) prepared from Chinese hamster ovary cells overexpressing glutathione *S*-transferase α (CHO-Chl^r; Ref. 21), kindly provided by Dr. A. G. Hall (University of Newcastle Upon Tyne, United Kingdom). Under the conditions used (500 μM melphalan, 12 μM [^{3}H]GSH) the monochloro-monoglutathionyl conjugate was the main reaction product. After HPLC separation on a C_{18} Hypersil column, using a water/acetonitrile gradient, the identity of the compound was established using ESI-MS/MS (22).

Synthesis of Glucuronides. Glucuronosyl [^{3}H]etoposide was synthesized from [^{3}H]etoposide and UDPGlcA using UDPGlcA β-D-glucuronosyltransferase from bovine liver microsomes (23). [^{3}H]Etoposide was dissolved in 50 mM Tris/HCl (pH 7.4) containing 10 mM $MgCl_2$, 10 mM UDPGlcA, and 1.5 milliunits glucuronosyltransferase. The incubation was terminated after 60 min at 37°C with the addition of ethanol. Precipitated protein was removed, and the supernatant was subjected to HPLC using a C_{18} Hypersil column and a linear water/acetonitrile gradient. The identity of the metabolite formed was established by ESI-MS/MS yielding a molecular mass of 764 daltons corresponding to a monoglucuronide of etoposide, which was described earlier as a major etoposide metabolite in bile (24).

The ^{14}C-labeled 6α-glucuronosylhyodeoxycholate was synthesized enzymatically from hyodeoxycholate and UDP-[^{14}C]GlcA in a reaction mixture containing 0.5 mM hyodeoxycholate, 0.25 mM UDP [^{14}C]GlcA, 5 mM $MgCl_2$, 50 mM Tris/HCl adjusted to pH 6.7, and glucuronosyltransferase from bovine liver microsomes (25). 5′-AMP (4 mM) was added to inhibit degradation of UDP [^{14}C]GlcA by nucleotide pyrophosphatase (EC 3.1.4.1). The glucuronide obtained was purified by HPLC on a C_{18} Hypersil column using a water/acetonitrile gradient and was coeluted in two different HPLC systems with standard 6α-glucuronosyl-[^{14}C]hyodeoxycholate kindly provided by Drs. H. Matern and S. Matern (Department of Medicine, Medical School of Aachen, Aachen, Germany).

Synthesis of Sulfate Conjugates. Sulfato[^{3}H]estradiol was purified from incubations of [^{3}H]estradiol with 3′-phosphoadenosine 5′-phosphosulfate and human liver homogenate in a buffer containing 0.25 M sodium acetate adjusted to pH 5.5. The product was reconverted to [^{3}H]estradiol by incubation with arylsulfatase from Helix pomatia (EC 3.1.6.1) and its molecular mass calculated from ESI-MS/MS corresponded to a monoconjugate of estradiol with sulfate. The fragmentation spectra indicated the conjugate to be 3α-sulfatoestradiol.

3α-Sulfatolithocholyl [2′-^{3}H]taurine was synthesized as described (26).

Preparation of Plasma Membrane Vesicles from HeLa Cells. HeLa cells transfected with the pRc/CMV vector (HeLa C1 cells) or with the vector containing the *MRP*-coding sequence (HeLa T5 cells) were kindly provided by Drs. S. P. C. Cole and R. G. Deeley (Queen's University, Kingston, Ontario, Canada). Cells were selected for their ability to grow in the presence of G418 (Geneticin), as described (17), and were cultured in RPMI 1640 medium with 10% FCS in a humidified incubator (5% CO_2, 37°C). Cells were harvested from the cultures by centrifugation, and plasma membrane vesicles were prepared as described previously (4).

Transport Studies in Membrane Vesicles. ATP-dependent transport of ^{3}H- or ^{14}C-labeled compounds into membrane vesicles was measured by rapid filtration (27) or in the case of hydrophobic substrates by centrifugation of the vesicles through a gel matrix using Nick spin columns (28). Membrane vesicles (20 μg protein) were incubated in the presence of 4 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg/ml creatine kinase, and labeled substrate in an incubation buffer containing 250 mM sucrose and 10 mM Tris/HCl (pH 7.4). The final incubation volume was 110 μl. The substrate and inhibitor concentrations are given in the respective legends to Figs. 1–4. In the rapid filtration procedure, the aliquots were diluted in 1 ml ice-cold incubation buffer and immediately filtered through nitrocellulose filters (0.2-μm pore size), which were presoaked in incubation buffer, and rinsed twice with 5 ml incubation buffer. Filters were dissolved in liquid scintillation fluid and counted for radioactivity. In the centrifugation procedure, Nick spin columns (1 g Sephadex G-50/2 ml) were prepared by rinsing with 250 mM sucrose, 10 mM Tris/HCl (pH 7.4), and centrifuged at 400 × *g* and at 4°C for 4 min before use. Aliquots of the incubations were diluted in 80 μl ice-cold incubation buffer and immediately loaded onto Sephadex G-50 columns. The columns were rinsed with 100 μl incubation buffer and centrifuged at 400 × *g* and at 4°C for 4 min. The effluents were collected and assayed for the vesicle-associated radioactivity (28). In control experiments, ATP was replaced by an equal concentration of the nonhydrolyzable ATP analogue AMP-PCP or by 5′-AMP. Rates of net ATP-dependent transport were calculated by subtracting values obtained in the presence of AMP-PCP or 5′-AMP as a blank from those in the presence of ATP.

RESULTS

Transport of Glutathione-derived *S*-Conjugates. As shown in Table 1, not only the glutathione conjugate [^{3}H]LTC_4, but also its cysteinylglycine metabolite [^{3}H]LTD_4 and its cysteinyl metabolite [^{3}H]LTE_4, as well as the mercapturate [^{3}H]LTE_4NAc were transported at low concentrations (50 nM) into membrane vesicles from MRP-overexpressing HeLa T5 cells. Under the same conditions, the related eicosanoids [^{3}H]LTB_4, [^{3}H]prostaglandin D_2, and [^{3}H]thromboxane B_2 were not transported in an ATP-dependent manner. For glutathione *S*-conjugates derived from xenobiotics, exemplified by DNP-[^{3}H]SG and by the mono[^{3}H]glutathionyl conjugate of the alkylating agent melphalan, significant rates of ATP-dependent transport were detected at low substrate concentrations (200 nM; Fig. 1 and Table 1).

ATP-dependent [^{3}H]GSSG transport, also mediated by MRP, reached a half-maximal rate only at about 100 μM (Tables 1 and 2). The efficiency of MRP-mediated [^{3}H]LTC_4 transport, calculated by the V_{max}:K_m ratio, was 9- and 145-fold higher than that of DNP-[^{3}H]SG and [^{3}H]GSSG, respectively (Table 2).

Transport of Glucuronidated and Sulfated Compounds. As indicated by the absent or reduced secretion into bile of GY/TR$^-$ mutant rats, LTC_4 shares a common canalicular export system with a variety of organic anions including dianionic bile salt conjugates (9, 10, 12). Therefore, [6α-^{14}C]glucuronosylhyodeoxycholate and 3α-sulfatolithocholyl [^{3}H]taurine were synthesized, and their substrate properties for MRP-mediated transport examined. As shown in Fig. 2, HeLa T5 membrane vesicles exhibited ATP-dependent transport of both bile salt conjugates. AMP-PCP instead of ATP served as a control and indicated a small time-dependent increase in vesicle-associated radioactivity (Fig. 2, *left panels*). In HeLa C1 control membranes, a low rate of ATP-dependent transport was observed with the sulfated bile salt, especially within the first 5 min of incubation. However, the vesicle-associated radioactivity at 5 and 15 min was significantly higher in the HeLa T5 membranes ($P < 0.001$ by Student's *t* test). Another endogenous steroid conjugate, 17β-glucuronosyl [^{3}H]estradiol, proved to have a relatively high affinity for MRP with a K_m value of 1.5 μM (Fig. 3; Tables 1 and 2). Native [^{3}H]estradiol as well as 3α-sulfato[^{3}H]estradiol and the monoanionic bile salt [^{3}H]cholyltaurine ([^{3}H]taurocholate) showed no ATP-dependent increase in vesicle-associated radioactivity at concentrations up to 5 μM. Glucuronides of drugs, exemplified by glucuronosyl [^{3}H]etoposide, were potential MRP substrates, at least at higher concentrations (Fig. 4). In contrast, native [^{3}H]etoposide, at concentrations up to 10 μM, was not a substrate for MRP-mediated transport.

Inhibition of MRP Function by MK 571. The LTD_4 receptor antagonist MK 571 is a potent inhibitor of MRP-mediated [^{3}H]LTC_4 transport (Ref. 4; Table 3). As demonstrated in Fig. 4 and Table 3, the ATP-dependent transport of glucuronosyl [^{3}H]etoposide, glucuronosyl [^{3}H]estradiol, and [^{3}H]GSSG into membrane vesicles from the *MRP*-transfected HeLa T5 cells was competitively inhibited with K_i values of <1 μM. The inhibitory efficiency of MK 571, estimated by the K_m:K_i ratio, was inversely related to the substrate affinity in the following order: [^{3}H]GSSG ≫ glucuronosyl [^{3}H]estradiol > [^{3}H]LTC_4 (Table 3).

Transport Studies with Anthracyclins and the *Vinca* Alkaloid Vinblastine. Our assay conditions for transport of [^{3}H]daunorubicin, [^{14}C]doxorubicin, and [^{3}H]vinblastine were optimized in canalicular

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Table 1 *Substrates of MRP-mediated transport*

Membrane vesicles from HeLa T5 cells were incubated with the ^{3}H- or ^{14}C-labeled compound (see "Materials and Methods") at the indicated concentration. The rates of net ATP-dependent transport were calculated as described in the legend to Fig. 1. The transport rates obtained with the HeLa C1 control membranes are given as percentage of HeLa T5 membrane transport. Data represent mean values ± SD from at least four determinations.

Substrate	Concentration	ATP-dependent transport	
		MRP-transfected HeLa T5 (pmol × mg protein^{-1} × min^{-1})	Control HeLa C1 (% of HeLa T5)
Cysteinyl leukotrienes			
LTC_4	50 nM	55 ± 5	<10
LTD_4		15 ± 2	<10
LTE_4		8 ± 1	<10
LTE_4NAc		3 ± 1	<10
Glutathione conjugates			
LTC_4	200 nM	86 ± 7	23 ± 3
DNP-SG		25 ± 3	<10
Monochloro-monoglutathionyl melphalan		2 ± 0.3	<10
GSSG		<0.5	
GSSG	100 μM	230 ± 11	<5
Glucuronides			
17β-Glucuronosyl estradiol	200 nM	3 ± 0.4	22 ± 2
	5 μM	74 ± 9	32 ± 5
6α-Glucuronosylhyodeoxycholate		14 ± 2	23 ± 11
Glucuronosyl etoposide	10 μM	94 ± 20	23 ± 5
Sulfate conjugate			
3α-Sulfatolithocholyltaurine	5 μM	27 ± 3[a]	28 ± 7[a]

[a] Calculated from the transport within 15 min (Fig. 2, *lower panels*).

membrane vesicles from rat liver which exhibit P-glycoprotein-mediated, ATP-dependent transport (28). The rapid filtration method was replaced in this study by centrifugation of the vesicles through a gel matrix to minimize unspecific binding. Under these conditions, [^{3}H]daunorubicin and [^{3}H]vinblastine, at a concentration of 10 μM, showed an ATP-dependent increase of canalicular membrane vesicle-associated radioactivity with initial rates of 235 and 66 pmol × mg protein^{-1} × min^{-1}, respectively. Transport of labeled anthracyclins and [^{3}H]vinblastine was investigated under identical conditions in membrane vesicles from the *MRP*-transfected HeLa T5 cells and from the HeLa C1 control cells. Significant differences were neither observed between measurements in the presence of ATP and AMP-PCP, nor between measurements in membrane vesicles from MRP-overexpressing and control cells. These results indicate that daunorubicin, doxorubicin, and vinblastine are not direct substrates for MRP-mediated transport in membrane vesicles.

DISCUSSION

The MRP pump confers multidrug resistance in cells transfected with an MRP expression vector (17, 29). An active ATP-dependent export of cytotoxic drugs from cancer cells was formerly exclusively related to overexpression of the *MDR1*-encoded P-glycoprotein that extrudes compounds which are hydrophobic and, at physiological pH, mostly cationic (for review, see Ref. 30). Another ATP-dependent transport protein, functionally known for many years, is the conjugate export pump in the canalicular membrane of hepatocytes (9), also termed multispecific organic anion trans-

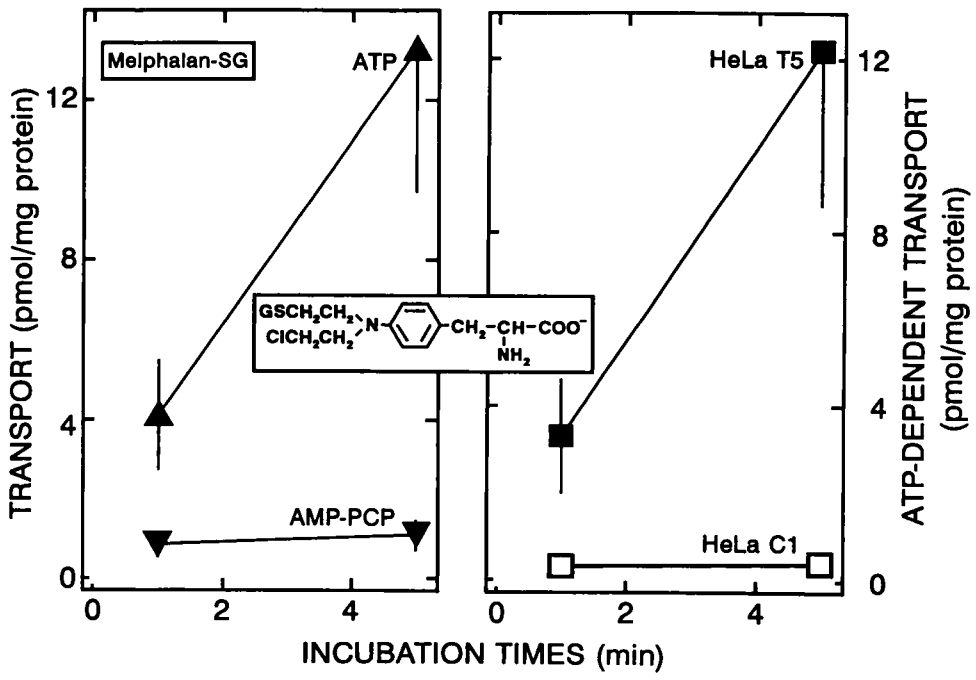

Fig. 1. MRP-mediated transport of melphalan conjugated with [^{3}H]glutathione. Transport of monochloro-mono[^{3}H]glutathionylmelphalan (*Melphalan-SG*; 200 nM) was studied in membrane vesicles from *MRP*-transfected HeLa T5 cells as well as in HeLa C1 control cells. Vesicle suspensions were incubated with monochloro-mono[^{3}H]glutathionylmelphalan in the presence of 4 mM ATP (▲) or its nonhydrolyzable analogue AMP-PCP (▼, *left panel*). The rates of net ATP-dependent transport into membrane vesicles from HeLa T5 (■) cells and from HeLa C1 cells (□) were calculated by subtracting the blank values obtained with AMP-PCP from those obtained in the presence of ATP (*right panel*). *Points*, mean values from seven experiments; *bars*, SD.

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Table 2 *Efficiency of different substrates for MRP-mediated transport*

The kinetic constants K_m and V_{max} were calculated from double reciprocal plots. The transport efficiency is expressed as $V_{max}:K_m$ ratio. Data represent mean values from at least three experiments with triplicate determinations.

Substrate	K_m (μM)	V_{max} (pmol × mg protein^{-1} × min^{-1})	$V_{max}:K_m$ (ml × mg^{-1} × min^{-1})
LTC_4	0.097 ± 0.02	100 ± 20	1031
DNP-SG	3.6 ± 0.7	409 ± 51	114
17β-Glucuronosyl estradiol	1.5 ± 0.3	63 ± 15	42
GSSG	93 ± 26	659 ± 184	7.1

porter (12), or glutathione *S*-conjugate export pump (14). Well-characterized substrates of this transporter are glutathione conjugates including glutathione disulfide (10, 20, 31, 32). The deficiency of this transporter was described in GY/TR$^-$ mutant rats (for review, see Ref. 12). These mutants selectively lack the canalicular Mrp isoform in hepatocytes (9). In addition to glutathione-derived conjugates, several glucuronidated as well as sulfated compounds were proposed as substrates for this export pump on the basis of their absence from bile of GY/TR$^-$ rats (for review, see Ref. 12). Using isolated plasma membrane vesicles from rat hepatocytes, an ATP-dependent transport, which is deficient in GY/TR$^-$ mutants, has been demonstrated for glucuronate conjugates of bilirubin (33), *p*-nitrobenzene (34), and nafenopin (23) in normal liver. The excretion of sulfate conjugates of taurolithocholate, glycolithocholate, and taurochenodeoxycholate is considerably impaired in GY/TR$^-$ rats, whereas the excretion of their unsulfated counterparts is normal (16). As shown in Fig. 2, ATP-dependent transport of 6α-glucuronosylhyodeoxycholate and 3α-sulfatolithocholyltaurine was detected in membrane vesicles from MRP-overexpressing HeLa T5 cells. In contrast, MRP-mediated transport was not detectable with cholyltaurine which is transported across the canalicular membrane by an export pump different from the one deficient in GY/TR$^-$ rats (12, 13, 28). Among the

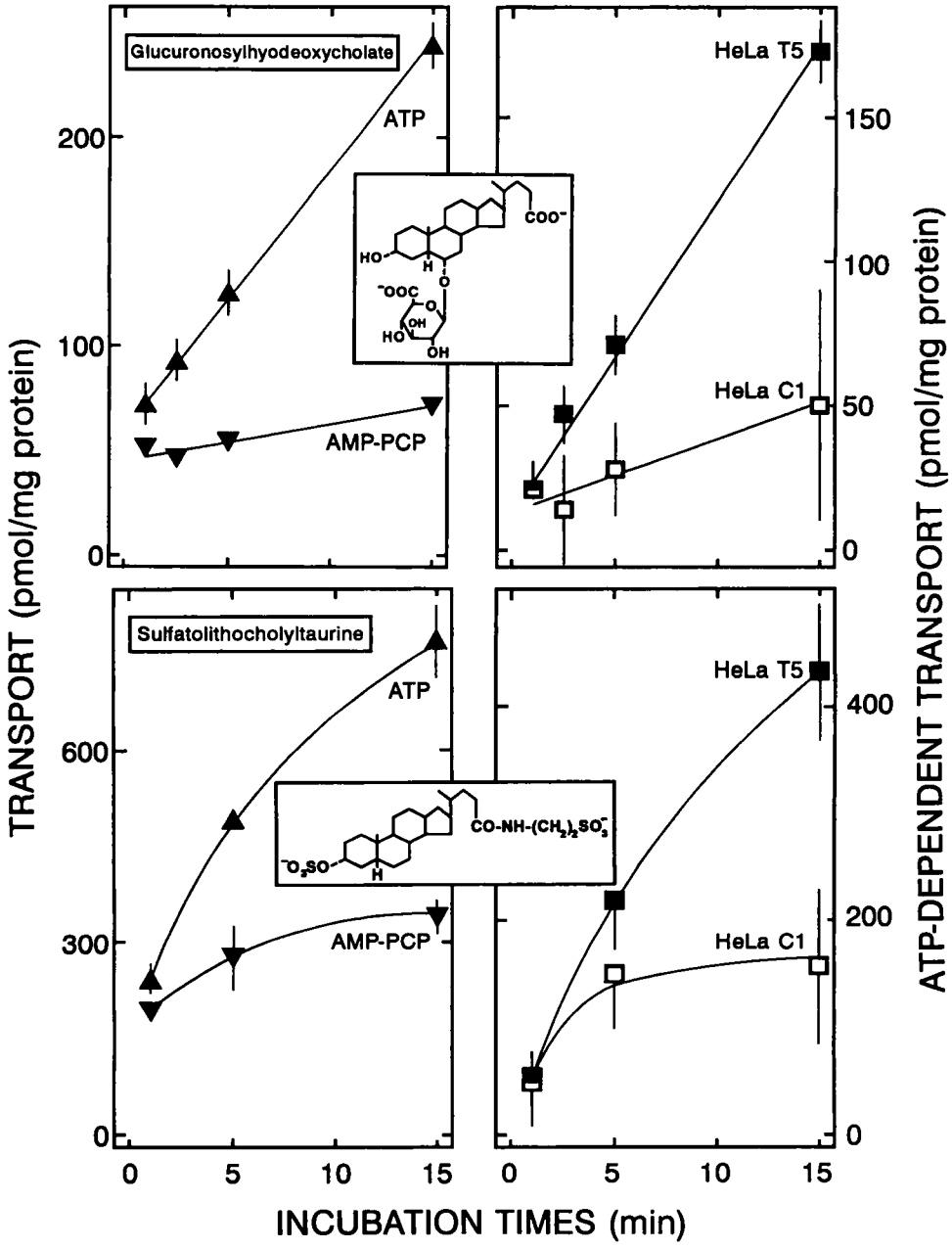

Fig. 2. MRP-mediated transport of dianionic bile salt conjugates. Transport of [6α-^{14}C]glucuronosylhyodeoxycholate (5 μM; *upper panels*) and 3α-sulfatolithocholyl[^{3}H]taurine (5 μM; *lower panels*) was studied in membrane vesicles from *MRP*-transfected HeLa T5 cells as well as in HeLa C1 control cells in the presence of 4 mM ATP (▲) or its nonhydrolyzable analogue AMP-PCP (▼, *left panels*). The rates of net ATP-dependent transport of both bile salt conjugates into membrane vesicles from HeLa T5 (■) cells as well as from HeLa C1 cells (□) are presented (*right panels*). *Points*, mean values from four experiments; *bars*, SD.

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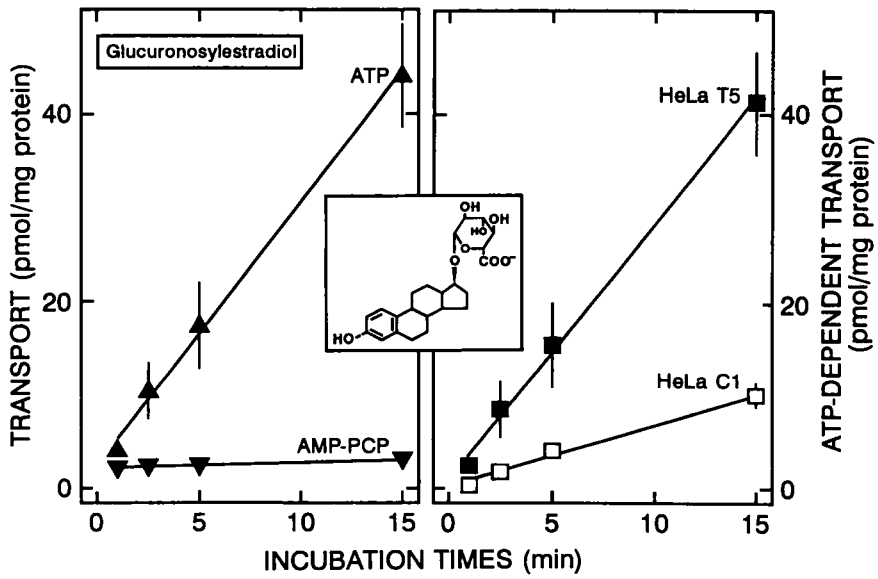

Fig. 3. ATP-dependent transport of glucuronidated estradiol in membrane vesicles from *MRP*-transfected (HeLa T5; ■) and control (HeLa C1; ○) cells. Transport assays with 200 nM 17β-glucuronosyl [^{3}H]estradiol were performed in the presence of ATP (▲) or AMP-PCP (▼; *left panel*) and the rate of ATP-dependent transport (*right panel*) was calculated as described in the legend to Fig. 1. *Points*, mean values from four experiments; *bars*, SD.

substrates without a glutathione moiety tested in the present study, 17β-glucuronosyl estradiol turned out to be a MRP substrate transported with a high affinity (Tables 1–3). Based on its reduced accumulation in MDR1-overexpressing cells, 17β-glucuronosyl estradiol was considered a physiological substrate for MDR1 P-glycoprotein (35). Furthermore, ATP-dependent transport of 17β-glucuronosyl estradiol by rat canalicular membranes was demonstrated (35), but the presence of the canalicular isoform of Mrp in the bile canalicular membrane as well as a possible co-overexpression of MRP with MDR1 P-glycoprotein in drug-selected cells were not considered at that time. A glucuronide of the anticancer drug etoposide was another monoanionic amphiphilic compound recognized as a substrate for ATP-dependent transport by MRP (Fig. 4). The formation and proposed structure of this etoposide metabolite has been described earlier (24).

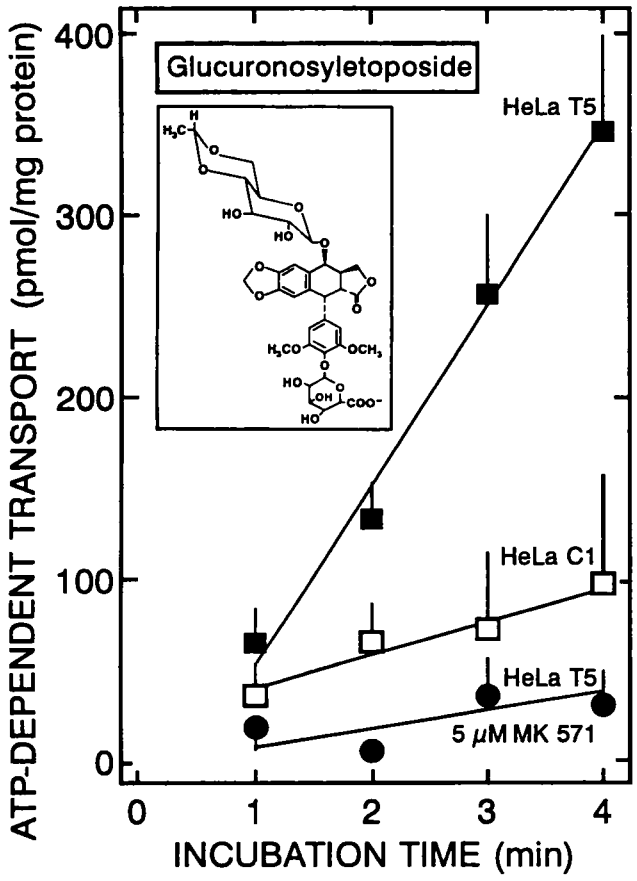

Fig. 4. ATP-dependent transport of glucuronidated etoposide and its inhibition by the LTD_4 receptor antagonist MK 571. Membrane vesicles from HeLa T5 (■) or HeLa C1 (□) cells were incubated with 10 μM glucuronosyl [^{3}H]etoposide, and the rates of ATP-dependent transport were calculated from the difference in transport in the presence of ATP and AMP-PCP. In addition, transport assays with HeLa T5 membrane vesicles were performed in the presence of 5 μM MK 571 (●). *Points*, mean values from four experiments; *bars*, SD. *Inset*, structure of a glucuronide of etoposide (24).

It has long been known that cancer cells resistant to alkylating agents such as melphalan may contain increased levels of glutathione and that their resistance can be reverted by glutathione depletion (36, 37). A similar reverting effect was observed in MRP-overexpressing cells resistant to anthracyclins, vincristine, and etoposide after inhibition of GSH biosynthesis by buthionine sulfoximine (38–42). Glutathione conjugates of melphalan can be isolated (43) and can serve as MRP substrates, although with a lower transport rate than glutathione conjugates possessing a more hydrophobic moiety (Fig. 1 and Table 1). For anthracyclins, glutathione may play a role in the removal of the superoxide radicals generated by these anticancer drugs (44). However, a stable covalent conjugate of anthracyclins with glutathione could not be isolated thus far. Our results indicate that daunorubicin, doxorubicin, or vinblastine are not direct substrates for MRP.

It is too early at this time to define the common structural features of substrates transported by MRP, except that they are amphiphilic anions with at least one negatively charged group. However, merely being hydrophobic and possessing an anionic group are not sufficiently distinctive characteristics to define substrates for this export pump, because several amphiphilic anions used in the present study were not transported by MRP. On the other hand, GSSG is a substrate for MRP without having a sizable hydrophobic domain (Table 2).

The finding that MRP confers resistance to antimonial and arsenical oxyanions (2, 42) suggests that, in addition to covalent conjugates, noncovalent complexes with GSH also may be substrates for MRP. Moreover, amphiphilic substrates may be transported in a complex comprising hydrophobic and anionic moieties, each exposed to appropriate binding sites of MRP.

The present study demonstrates that the human *MRP* gene en-

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Table 3 *Inhibitory efficiency of MK 571 for MRP-mediated transport of different substrates*

The inhibition constants K_i were calculated from double reciprocal plots. The type of inhibition was competitive with respect to each of the three substrates. K_i values were calculated from one experiment with triplicate determinations and were related to the respective K_m value given in Table 2.

Substrate	K_i (μM)	K_m/K_i
LTC_4	0.6	0.16
17β-Glucuronosyl estradiol	0.7	0.46
GSSG	0.6	155

codes an export pump with a broad specificity which is very similar to the one ascribed to the ATP-dependent conjugate export pump in the rat hepatocyte canalicular membrane. In comparison to the large number of highly specific drug-metabolizing and -conjugating enzymes involved in detoxification processes, the broad specificity of MRP points to a small number of export pumps excreting endogenous and xenobiotic substances and their conjugates. Knowledge of physiological and xenobiotic MRP substrates is a prerequisite for the design of transport inhibitors, or derivatives of anticancer drugs which are not easily pumped out from tumor cells by MRP, and may thus serve to counteract MRP-mediated multidrug resistance.

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