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Characterization of Drug Transport by the Human Multidrug Resistance Protein 3 (ABCC3)*

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We have characterized the substrate specificity and mechanism of transport of the human multidrug resistance-associated protein 3 (MRP3). A murine fibroblast-like cell line generated from the kidneys of mice that lack Mdr1a/b and Mrp1 was retrovirally transduced with MRP3 cDNA. Stable clones overproducing MRP3 were resistant to the epipodophyllotoxins etoposide and teniposide but not to vincristine, doxorubicin, and cisplatin, drugs suggested to be MRP3 substrates by others. The resistance to etoposide was associated with reduced cellular accumulation and enhanced efflux of this drug and was not affected by depleting cells of glutathione but was inhibited by several common organic anion transport inhibitors. Membrane vesicles from infected insect cells expressing *MRP3* mediated ATP-dependent transport of estradiol 17-β-D-glucuronide, leukotriene C_4, dinitrophenyl *S*-glutathione but not glutathione itself, and etoposide glucuronide, a major metabolite of etoposide *in vivo*. The transport of estradiol 17-β-D-glucuronide by MRP3 was inhibited in a concentration-dependent manner by both etoposide and methotrexate. Even though etoposide glucuronide is an excellent substrate for MRP3, this compound is not involved in the etoposide resistance of our MRP3 cells, as these cells extrude unmodified etoposide rather than etoposide glucuronide.

Resistance to multiple anticancer drugs is an impediment to the successful treatment of human malignancies. "Classical" multidrug resistance of cancer cells, characterized by resistance to structurally and functionally unrelated compounds, is caused by overexpression in tumor cells of drug efflux pumps belonging to the ATP binding cassette (ABC) family of membrane transporters (1). Specifically, overexpression of the 170-kDa MDR1 P-glycoprotein (2), of members of the multidrug resistance protein (MRP)[1] family, typified by the 190-kDa MRP1 (3), and of breast cancer resistance protein (4) have all been associated with the multidrug resistance phenotype in tumor cell lines.

The MRP family of membrane transporters consists of 7 members known as MRP1–7 (5). MRPs function as organic anion transporters and have been shown to extrude a remarkably diverse array of substrates including anticancer drugs (5), sulfate, glucuronide, or glutathione (GSH) conjugates of diverse compounds (6), cyclic peptides (7), monophosphate nucleotides (8, 9), and cyclic nucleotides (10). To date, only the physiological roles of MRP1 and MRP2 have been elucidated. Mrp1 is the high affinity leukotriene C_4 transporter (LTC_4) (6, 11) and can protect some tissues from etoposide-induced cytotoxic damage (12). MRP2 is the major organic anion transporter in the canalicular membrane of hepatocytes (13, 14). The physiological roles of the remaining MRPs have yet to be determined.

Among the MRP family members, MRP3 shares the highest amino acid sequence identity (58%) with MRP1 (15), and both localize to the basolateral membranes of polarized cells (16, 17). In humans, MRP3 expression is highest in the adrenal glands and the intra-hepatic bile ducts followed by moderate to low expression in the small intestine, kidney, and pancreas (18). MRP3 is highly up-regulated in the livers of rats made cholestatic by bile duct ligation (19–21) or in cholestatic human liver (16). These findings suggest that MRP3 plays a role in the removal of toxic organic anions from the liver under cholestatic conditions. Support for this notion came from biochemical studies using a vesicular transport system with vesicles enriched for rat Mrp3 (22). Using this system Hirohashi *et al.* (22) establish that rat Mrp3 transports several organic anions with a preference toward the glucuronidated ones. A subsequent study by the same group (23) demonstrated that rat Mrp3 transports the bile salts taurocholate, glycocholate, taurochenodeoxycholate 3-sulfate, and taurolithocholate-3-sulfate, thereby linking MRP3 to a possible role in the entero-hepatic circulation of bile salts. Comparable results were recently obtained with human MRP3, with the exception that no taurocholate transport was detected (24).

Because MRP1 has a broad substrate specificity for anticancer drugs, the possible role of MRP3 in drug resistance has been extensively studied. Kool *et al.* (16) used 2008 ovarian carcinoma cells overproducing MRP3 to demonstrate low level resistance to the epipodophyllotoxins etoposide and teniposide but not to podophyllotoxin itself. Additionally, these cells were highly resistant to short term incubations with high concentrations of methotrexate (MTX), in agreement with the MTX transport found using the vesicular systems (23, 24). Similar

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[1] The abbreviations used are: MRP, multidrug resistance-associated protein; MTX, methotrexate; VCR, vincristine; GSH, glutathione; PBS, phosphate-buffered saline; BSO, buthionine sulfoximine; HBS, Hepes-buffered saline solution; Sf9, *S. frugiperda*; $E_2$17βG, estradiol 17-β-D-glucuronide; LTC_4, leukotriene C_4; DNP-SG, 2,4-dinitrophenyl *S*-glutathione; HPLC, high performance liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid.

results were obtained by König *et al.* (17), whereas Zeng *et al.* (25) additionally found low level resistance to vincristine (VCR). This suggests that the substrate specificity of MRP3 might be broader than initially thought. Support for this idea comes from studies by Young *et al*. (26, 27). By screening a panel of lung cancer cells lines, they found a strong correlation between MRP3 expression and resistance to doxorubicin and a weaker although significant correlation with resistance to vincristine, etoposide, and cisplatin.

An additional unresolved problem is the mechanism by which MRP3 transports drugs that are not negatively charged under physiological conditions, such as etoposide. In the case of MRP1 (28) and MRP2 (29) there is now strong evidence that *Vinca* alkaloids are co-transported with GSH. Transport of some organic substrates is even stimulated by GSH (30). MRP1 and -2 are also able to transport GSH by itself, albeit with low affinity. In contrast, MRP3-overproducing cells do not excrete GSH (16), and therefore, the role of GSH in MRP3-mediated drug resistance is not yet clear. In this paper we analyze the substrate specificity of MRP3 and its mechanism of drug transport in MRP3-overproducing fibroblasts derived from the kidneys of mice that have a targeted disruption of *Mdr1a/b* and *Mrp1* (31) and in vesicular transport assays.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Etoposide (411 or 280 mCi/mmol) was purchased from Moravek (Brea, CA). [^{3}H]GSH (50 Ci/mmol), [^{3}H]LTC_4 (146 Ci/mmol), and estradiol 17-β-D-glucuronide ($E_2$17βG) (44 Ci/mmol) were obtained from PerkinElmer Life Sciences. Unlabeled and ^{3}H-labeled 2,4-dinitrophenyl *S*-glutathione (DNP-SG) were synthesized enzymatically using glycine-2-[^{3}H]GSH (PerkinElmer Life Sciences) and 1-chloro-2,4-dinitrobenzene and glutathione *S*-transferase (Sigma) as described previously (32). Unless indicated, all other chemicals and drugs were obtained from Sigma. Solvents used for HPLC analysis were of HPLC grade.

Cell Lines—Fibroblasts derived from the kidneys of mice that have a homozygous deletion of Mdr1a/b, and Mrp1 cells (31) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100 units/ml penicillin/streptomycin. Cells were grown at 37 °C with 5% CO_2 under humidifying conditions. Cell lines were regularly checked for the absence of mycoplasma infection.

Cloning and Transfection of Cell Lines—A cDNA insert encoding MRP3 was excised from pGEM7-MRP3 (16) using *Aat*II and *Sph*I, blunted, and cloned into the *Eco*RV site of the retroviral pBabePURO vector (33). The resulting pBabePURO-MRP3 vector was transfected into the amphotropic retroviral packaging cell line Phoenix (34). The virus particles produced were used to transduce fibroblasts derived from the kidneys of mice that lack Mdr1a/b and Mrp1. As control, the same line was transduced with virus particles that contained no MRP3 cDNA insert. After 48 h, the cells were split, and stable clones were selected with 2 μg of puromycin/ml. Resistant clones were analyzed for MRP3 overproduction by immunoblotting. No endogenous murine Mrp3 was detected either in the parental V1 clone or in transfectant MRP3 38 using a rabbit antiserum reacting with mouse Mrp3 but not human MRP3 (not shown).

To generate recombinant baculovirus, the MRP3 cDNA was cloned into pBlueBac4.5 (Invitrogen, Carlsbad, CA). Baculovirus for human MRP3 expression was generated using the Bac-N-Blue expression system (Invitrogen) according to the manufacturer's protocol.

Protein Analysis and Immunohistochemistry—Total cell lysates were prepared in a hypotonic lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) supplemented by a mixture of protease inhibitors used at the dilution recommended by the manufacturer (Roche Molecular Biochemicals). Samples containing varying amounts of protein were separated on a 7.5% SDS-polyacrylamide gel. Subsequently, gels were blotted overnight in a tank blotting system. MRP3 was detected using the monoclonal antibody M_3II-9 (1:250) followed by a rabbit anti-mouse horseradish peroxidase conjugate (1:1000) and visualized with chemiluminescence (ECL, Amersham Pharmacia Biotech). Mdr1a/b Pgp and Mrp1 were detected using the monoclonal antibodies C219 and MRP-R1, respectively. For immunolocalization of MRP3, cells were grown overnight on glass slides. MRP3 was detected using M_3II-9 (1:50) as the primary antibody and Alexa 488-labeled goat anti-mouse IgG (1:100) as the secondary antibody as described (16).

Growth Inhibition Assays—The drug sensitivity of cells was determined using growth inhibition assays under continuous long term drug exposure. Cells were plated at a density of 1000 cells/well in triplicate in a 96-well plate. After 24 h, drugs in varying concentrations were added to each well. Incubations were continued for an additional 72 h. For studying the effect of GSH depletion on drug sensitivity cells were incubated for 24 h with 50 μM buthionine sulfoximine (BSO) before the addition of drug. At the end of the assay, the medium was removed from the plates, and the plates were frozen at −80 °C for at least 4 h. Subsequently the plates were thawed, and the total number of cells was determined with fluorescence by using the CyQuant cell proliferation assay kit (Molecular Probes, Leiden, The Netherlands). Fluorescence was measured with a CytoFluor 4000 fluorescence plate reader (Applied Biosystems, Norwalk, CT). The relative resistance was calculated as the ratio of inhibitory concentration IC_{50} (where 50% of the cells survive) of the resistant cell line to the IC_{50} of the parental cell line.

[^{3}H]Etoposide Accumulation Assays—One day before the assay, cells were plated at a density of 1×10^6/well. The following day, cells were washed twice with pre-warmed phosphate-buffered saline (PBS) and incubated with 1 ml of 2 μM [^{3}H]etoposide for 1 h at 37 °C. Subsequently, the cells were washed twice with ice-cold PBS, trypsinized, and transferred to a counting vial. Radioactivity was measured by liquid scintillation counting in a Packard 1900CA counter (Packard Instrument Co.). The capacity of several general organic anion inhibitors to inhibit the MRP3-mediated accumulation defect was studied by adding them at varying concentrations with the [^{3}H]etoposide to the incubation medium.

[^{3}H]Etoposide Efflux—Cells were plated at a density of 1×10^6/well 1 day before assay. The following day, cells were washed twice with pre-warmed PBS and incubated with 1 ml of Dulbecco's modified Eagle's medium without glucose supplemented with 10% dialyzed fetal calf serum, 10 mM sodium azide, 10 mM 2-deoxyglucose, and 2.5 μM [^{3}H]etoposide for 1 h at 37 °C. After this, the cells were washed twice with cold PBS, and efflux was initiated by the addition of prewarmed complete Dulbecco's modified Eagle's medium. Samples from the efflux medium were taken at various time points and measured using liquid scintillation counting.

GSH Assays—To determine intracellular GSH levels, 2×10^6 cells were plated/well. Medium was refreshed 24 h later. When the effect of BSO on intracellular GSH was studied, medium containing 50 μM BSO was used. After an additional 24 h the cells were washed twice with PBS, and intracellular GSH was determined as described (16). For studying the efflux of GSH, cells were plated as described above. The following day the cells were washed twice with pre-warmed Hepes-buffered saline solution, pH 7.4 (HBS), and incubated at 37 °C with 1 ml of HBS containing 500 μM acivicin to inhibit γ-glutamyltransferase activity. When the effect of etoposide on GSH efflux was studied, etoposide was included at a concentration of 34 μM. Samples were taken at different time points. After the last sample, the cells were washed twice with ice-cold HBS buffer, and intracellular GSH was determined as above.

Synthesis of [^{3}H]Glucuronosyl Etoposide—Synthesis of etoposide glucuronide was as described by others (6, 30) with several minor modifications. In brief, 0.2 mg of rat liver microsome preparations were incubated with 20 mM UDP-glucuronic acid, 10 mM $MgCl_2$, 50 mM MOPS, pH 7.4, and [^{3}H]etoposide (final concentration 100 μM) in a total volume of 200 μl at 37 °C for 2 h. The reaction was terminated by the addition of an equal volume of acetonitrile. After removal of protein, the reaction mix was fractionated by HPLC, and fractions containing [^{3}H]etoposide glucuronide were collected. These fractions were freeze-dried and resuspended in water. The identity of the metabolite was confirmed by mass spectrometry (Sciex, API3000) operating in the negative ion mode. The mass spectrum showed the highest mass fragment at *m/z* 763.4 ($[M\text{-}H]^-$) and ions at *m/z* 571.0 ($[M+H\text{-glucuronic acid}]^-$) with high intensity. Nonlabeled etoposide glucuronide was synthesized in a similar manner with the exception that dog liver microsomes (Gentest, Woburn, MA) were used. The identity of the metabolite was confirmed as above.

HPLC Analysis—HPLC analysis of [^{3}H]etoposide and potential metabolites was done as described by Hande *et al*. (35) with minor modifications. In brief, efflux medium was mixed with an equal volume of acetonitrile to precipitate proteins, vortexed for 1 min, and centrifuged at 10,000 rpm for 1 min. 50 μl of the resulting supernatant were injected on a 3.9 × 300-mm, μBondapak phenyl column (Waters, Milford, MA). Ultraviolet absorbance (254 nm) was monitored, and a mobile phase of water:acetonitrile:acetic acid (74:25:1) was used to separate etoposide from its potential metabolites. Eluate from the column was collected in 1-ml fractions. Radioactivity in the fractions was measured by liquid scintillation.

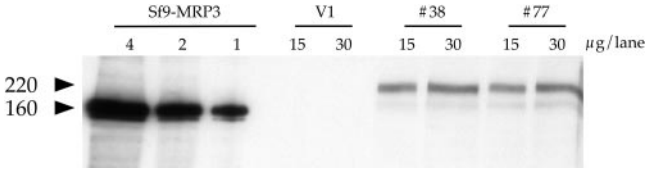

FIG. 1. **Immunoblot analysis of MRP3 expression in selected clones.** MRP3 expression in Sf9-MRP3 membranes and in selected transfected fibroblast clones is shown. SF9-MRP3 membranes containing 1–4 μg of protein or 15 or 30 μg of total cellular protein were loaded per *lane*, and MRP3 was detected using monoclonal antibody M_3II-9 (16).

Preparation of Membrane Vesicles—Spodoptera frugiperda (Sf9) cells were infected with MRP3-expressing baculovirus at a multiplicity of infection of 1. After incubation at 27 °C for 3 days, cells were harvested by centrifugation at 3000 rpm for 5 min. The pellet was resuspended in ice-cold hypotonic buffer (0.5 mM sodium phosphate, 0.1 mM EDTA, pH 7.4) supplemented with protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μM pepstatin) and incubated at 4 °C for 90 min. The suspension was centrifuged at 4 °C at 100,000 × *g* for 40 min, and the pellet was homogenized in ice-cold TS buffer (50 mM Tris·HCl, 250 mM sucrose, pH 7.4) using a tight-fitting Dounce homogenizer. After centrifugation at 500 × *g* at 4 °C for 10 min, the supernatant was centrifuged at 4 °C at 100,000 × *g* for 40 min. The pellet was resuspended in TS buffer and passed 20 times through a 27-gauge needle. The vesicles were dispensed in aliquots, frozen in liquid nitrogen, and stored at −80 °C until use.

Vesicular Transport Assays—The time- and concentration-dependent uptake of various substrates into MRP3-containing vesicles was studied by the rapid filtration method. Briefly, membrane vesicles containing 20 μg of protein were incubated with the indicated concentrations of substrate in 50 μl of TS buffer in the presence of 4 mM ATP or AMP, 10 mM $MgCl_2$, 10 mM creatine phosphate, and 100 μg of creatine kinase/ml. At the indicated time, the reaction mixture was diluted in 1 ml of ice-cold TS buffer and immediately filtrated through a pure cellulose filter (0.45-μm pore size). The filter was washed twice with 3 ml of ice-cold TS buffer, and the radioactivity retained on the filter was measured by liquid scintillation.

RESULTS

Cloning and Generation of MRP3 Transfectants—To generate stable clones that overproduce MRP3, the MRP3 cDNA was cloned into the retroviral vector pBabePURO. The virus particles that were produced were used to transduce fibroblasts derived from the kidneys of mice that lack Mdr1a/b and Mrp1. Clones that survived selection with 2 μg of puromycin/ml were expanded and analyzed by Western blotting for MRP3 overproduction. Two clones, MRP3 38 and MRP3 77 were used for further study (Fig. 1). In both clones, MRP3 was detected as a double band, presumably resulting from differential glycosylation. We have previously shown in other cells that treatment with tunicamycin, which blocks *N*-glycosylation, results in the two MRP3 bands shifting to one band which runs with higher mobility (16). Levels of MRP3 were stable and showed no decrease for at least 4 months. As expected from the genetic background from which the cell line was generated, expression of Mdr1a/1b or Mrp1 was not detectable (data not shown). As controls, clones were generated from cells transduced with the empty pBabePURO vector, and clone V1 was used as a reference in further experiments. All the clones had similar morphology, size, and cell volume (data not shown). Confocal laser-scanning microscopy was used to determine the subcellular localization of MRP3 in clones MRP3 38 and 77 (Fig. 2). Using the monoclonal antibody M_3II-9, we detected MRP3 predominantly in the plasma membranes of these clones and in areas of cell-cell contact. However, a portion of MRP3 was found intracellularly, similar to what was found previously with transfectants of the ovarian carcinoma cell line 2008 (16). No staining was seen in the V1 control clone.

Growth Inhibition Assays—Growth inhibition assays were performed with continuous drug exposure with two independent MRP3-overproducing clones. The list of compounds tested is summarized in Table I. High resistance was found only against etoposide and teniposide (Fig. 3). To study the effect of GSH depletion on MRP3-mediated etoposide and teniposide resistance, the cells were preincubated with BSO, an inhibitor of GSH biosynthesis, before the addition of drugs. Preincubation of the cells with 50 μM BSO (a nontoxic concentration, data not shown) resulted in a drastic decrease in intracellular GSH levels (Table I). This had no effect on the resistance of MRP3 cells to etoposide or teniposide (Table I).

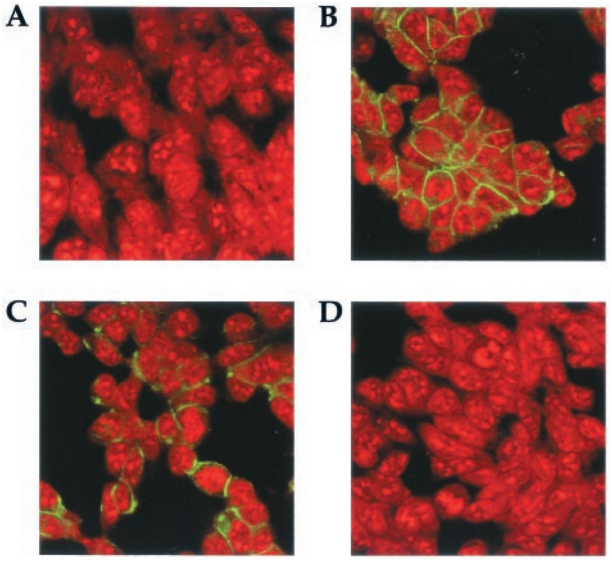

FIG. 2. **Immunolocalization of MRP3.** MRP3 was immunolocalized by primary monoclonal antibody M_3II-9 (1:50) followed by Alexa 488-labeled goat anti-mouse IgG (1:100). *A*, control clone, V1; *B*, MRP3 38; *C*, MRP3 77; *D*, MRP3 77, in the absence of the primary antibody.

[^{3}H]Etoposide Accumulation Assays—To further investigate the mechanism of MRP3-mediated etoposide drug resistance, we performed steady state drug accumulation experiments. Accumulation of [^{3}H]etoposide reached steady state levels after 1 h of incubation (data not shown), and therefore, all assays were conducted for this duration. Cells were incubated with 2 μM [^{3}H]etoposide at 37 °C, and the radioactivity retained intracellularly was measured. The V1 control clone accumulated 4.2 ± 0.4 pmol of [^{3}H]etoposide/1 × 10^6 cells, whereas MRP3 38, and MRP3 77 showed a substantial decrease in accumulation, accumulating 0.8 ± 0.1 and 1.3 ± 0.3 pmol of [^{3}H]etoposide/1 × 10^6 cells, respectively (n = 10). The results are summarized in Table II. The decrease in etoposide accumulation that clones MRP3 38 and 77 displayed allowed us to study the inhibitory capacity of several common organic anion transport inhibitors and drugs. As summarized in Table II, benzbromarone, sulfinpyrazone, probenecid, and indomethacin inhibited the MRP3-mediated accumulation defect in a dose-dependent manner at concentrations that were non-toxic to the cells. In contrast, ibuprofen at a concentration of up to 250 μM and 20 μM daunorubicin (data not shown) did not reverse the accumulation defect. MTX at concentrations of up to 5 mM had no effect on etoposide accumulation, but the cells were also resistant to this concentration of MTX, presumably because they lack an efficient uptake mechanism for this drug.

[^{3}H]Etoposide Efflux Assay—To obtain an estimate of the initial rate of MRP3-mediated etoposide transport we performed drug efflux experiments. Because our MRP3 clones accumulate less etoposide than our control clone, we had to use ATP-depleting conditions to load cells with [^{3}H]etoposide. Under these conditions, clones V1, MRP3 38, and MRP3 77

TABLE I
Drug resistance phenotype of MRP3 cells

1000 cells were plated in triplicate 24 h before the addition of drug. Cells were grown for an additional 72 h in the presence of varying concentrations of drug after which cellular growth was measured, and the concentration (±S.D.) that inhibited cells growth by 50% was determined (IC_{50}).

Drug	V1 IC_{50}	MRP3 38		MRP3 77		N[b]
		IC_{50}	RF[a]	IC_{50}	RF	
	nM	*nM*		*nM*		
Etoposide	102 ± 13	857 ± 47	8.4	703 ± 146	6.9	18
Teniposide	28 ± 4	128 ± 35	4.6	113 ± 31	4.1	9
Etoposide/BSO[c]	104 ± 14	801 ± 61	7.7	669 ± 108	6.4	3
Teniposide/BSO[c]	29 ± 6	136 ± 26	4.6	93 ± 26	3.2	3
Podophyllotoxin	16 ± 2	16 ± 2	1	ND[d]	ND	3
Doxorubicin	38 ± 14	39 ± 14	1	32 ± 4	0.8	3
Vincristine	2.9 ± 2.0	3.6 ± 2.9	1.2	2.4 ± 1.2	0.8	4
Daunorubicin	23 ± 4	25 ± 7	1.1	20 ± 0	0.9	3
SN-38	131 ± 49	160 ± 45	1.2	ND	ND	3
Cisplatin	6000 ± 2000	6400 ± 2500	1.1	5000 ± 1200	0.8	3

[a] RF, relative resistance factor of MRP3 cells calculated as the ratio of MRP3 IC_{50} to that of V1 IC_{50}.
[b] Number of independent experiments.
[c] Cells were pretreated for 24 h with 50 μM BSO before the addition of drugs. This resulted in a decrease of the intracellular GSH concentrations from 10.2 ± 1.3, 11.8 ± 1.6, 12.3 ± 1.9 to 0.8 ± 0.4, 1.6 ± 0.4, 1.5 ± 0.4 (nmol of GSH/mg of protein) for clones V1, MRP3 38, and MRP3 77, respectively.
[d] ND, not determined.

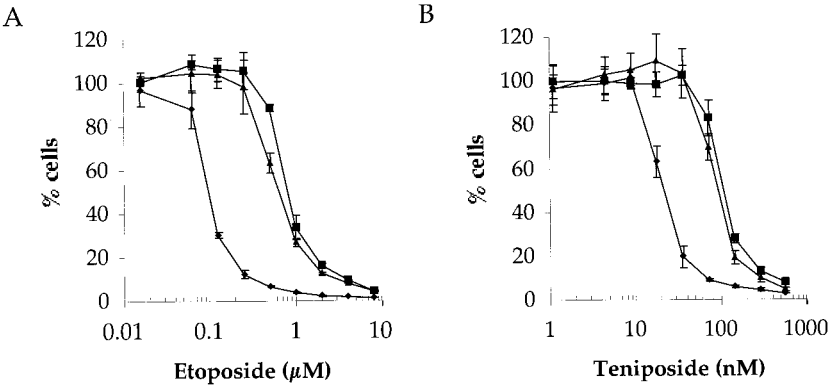

FIG. 3. **Drug resistance of MRP3 cells.** 1000 cells/well were plated in triplicate 24 h before the addition of drug. Cells were exposed to drug for 72 h after which cellular growth was quantified. A representative growth inhibition assay for etoposide (*A*) and teniposide (*B*) is shown. ◆, control, V1; ■, MRP3 38; ▲, MRP3 77. Each point represents the average ± S.E. of a measurement done in triplicate.

TABLE II
Steady state etoposide accumulation in MRP3-overproducing cells

Cells were incubated with 2 μM [^{3}H]etoposide for 1 h in the presence or absence of various inhibitors, and the intracellular radioactivity retained was determined. Each value is the average ± S.E. of experiments done in triplicate expressed as the percent of accumulation relative to control clone V1 in the absence of inhibitor.

Treatment	V1	MRP3 38	MRP3 77
Control	100 ± 0	18 ± 3	30 ± 8
Benzbromarone			
25 μM	105 ± 8	19 ± 3	26 ± 11
100 μM	110 ± 4	28 ± 3	37 ± 8
250 μM	97 ± 1	73 ± 7	67 ± 11
Indomethacin			
100 μM	88 ± 6	26 ± 4	31 ± 9
250 μM	84 ± 2	53 ± 3	56 ± 6
500 μM	76 ± 1	67 ± 2	64 ± 6
Probenecid			
0.5 mM	89 ± 19	30 ± 3	39 ± 8
1 mM	91 ± 14	41 ± 4	48 ± 6
5 mM	76 ± 9	59 ± 5	58 ± 5
Sulfinpyrazone			
0.5 mM	100 ± 1	27 ± 14	42 ± 4
1 mM	101 ± 2	50 ± 25	65 ± 0
2 mM	92 ± 1	83 ± 4	77 ± 1
Ibuprofen			
1 μM	101 ± 5	19 ± 1	25 ± 5
10 μM	105 ± 9	17 ± 2	22 ± 5
100 μM	99 ± 9	16 ± 1	23 ± 4

showed similar accumulation of [^{3}H]etoposide (18.9 ± 2.1, 16.3 ± 0.4, 16.7 ± 1.9 pmol/1 × 10^6 cells, respectively, n = 3). Upon initiation of efflux by the addition of complete medium, the fraction of etoposide recovered in the medium was significantly higher in both MRP3 clones than in the control clone (Fig. 4). In contrast, efflux in ATP depletion medium was equal in the MRP3 clones and the control line, demonstrating the ATP dependence of MRP3 transport (data not shown). The initial rate ratios for etoposide efflux of MRP3 38 and 77 were 1.7× and 1.5×, respectively, higher than that found in the control clone.

Analysis of GSH Transport by MRP3—The results in Table I indicate that MRP3 does not transport etoposide with GSH. We verified this by determining the GSH efflux from our cells in the absence or presence of etoposide. In the absence of drug, clones V1, MRP3 38, and MRP3 77 showed a similar low basal level efflux of GSH after 1 h (0.8 ± 0.4, 0.6 ± 0.3, and 0.6 ± 0.4 nmol GSH/mg of protein, respectively, n = 3), and this was not significantly increased in the presence of 34 μM etoposide (1.1 ± 0.3, 0.8 ± 0.4, and 0.7 ± 0.3 nmol GSH/mg of protein for clones V1, MRP3 38, and MRP3 77, respectively). In contrast, a clone that overproduces MRP1 (clone MRP1 2.2)[2] showed a 4-fold higher basal level of GSH efflux than MRP3 cells, and this efflux rate was 2-fold enhanced by 34 μM etoposide (data not shown).

The Ability of MRP3 Vesicles to Take Up GSH or Etoposide Glucuronide—To study direct transport of GSH by MRP3, we generated Sf9 membrane vesicles containing MRP3. These membrane vesicles contain 45-fold higher levels of MRP3 than

[2] N. Zelcer, T. Saeki, G. Reid, J. H. Beijnen, and P. Borst, unpublished information.

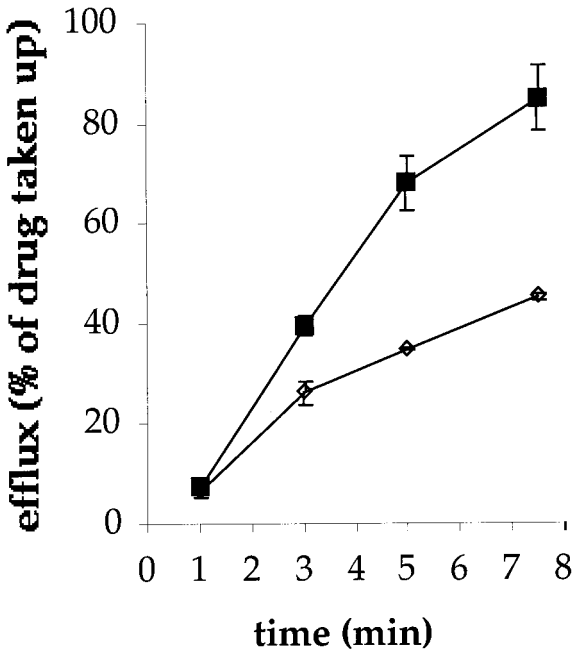

FIG. 4. **Etoposide efflux from MRP3 cells.** Cells were incubated with 2.5 μM [^{3}H]etoposide under ATP-depleting conditions for 1 h. Subsequently, efflux was initiated into complete medium, and samples were taken at the indicated times. Results from a representative etoposide efflux assay are presented. For clarity, only clone V1 and MRP3 38 are shown. ◇, control, V1; ■ MRP3 38. Each point represents the average ± S.E. of a measurement done in triplicate.

total cell lysates of the fibroblast clone MRP3 38 as determined by densitometry (Fig. 1). Uptake of GSH into these vesicles (Fig. 5*A*) was not higher than into control vesicles (Fig. 5B), in agreement with our results with intact cells. In contrast, Sf9 membrane vesicles containing MRP1 showed a significant time- and ATP-dependent uptake of GSH (Fig. 5*C*). Membranes from mammalian cells transfected with rat *Mrp3* or human *MRP3* cDNA constructs transported glucuronosyl derivatives with high affinity but glutathione conjugates poorly (22, 24). We also find this for the Sf9 membrane vesicles containing MRP3. Membranes containing MRP3 accumulate $E_2 17\beta G$ in an ATP-dependent manner (Fig. 6*A*) with K_m and V_{max} values of 17.7 ± 2.6 μM and 474 ± 63 pmol/mg of protein/min, respectively. The vesicles also accumulate LTC_4 and DNP-SG in an ATP-dependent manner at a level only 2–3 times higher than vesicles prepared from Sf9 cells infected with wild type baculovirus (Fig. 6, *B* and *C*), in agreement with results obtained by Zeng *et al.* (24) with mammalian cell vesicles. The ATP-dependent uptake of $E_2 17\beta G$ by MRP3-containing vesicles was inhibited by MTX and etoposide as well as by the same inhibitors used in the etoposide accumulation assays (Table III). The inhibition of vesicular transport by etoposide was not stimulated by including GSH in the reaction, indicating that GSH is not required for etoposide-mediated inhibition of MRP3 (data not shown).

Etoposide glucuronide is a major metabolite of etoposide *in vivo* (35) and is transported by MRP1 (6, 30). We therefore tested whether radioactive etoposide glucuronide enzymatically made with rat-liver microsomes is a substrate of MRP3. Fig. 6*D* shows that MRP3 membrane vesicles take up etoposide glucuronide at a substantial rate, even at the low substrate concentration (82.5 nM) used. Uptake is ATP-dependent and MRP3-specific; the background with this substrate is negligible at this low substrate concentration. Because the enzymatic synthesis of etoposide glucuronide with rat microsomes was inefficient in our hands, we used dog liver microsomes to prepare sufficient amounts of cold etoposide glucuronide to determine an approximate K_m for this substrate. Fig. 7 shows the concentration-dependent transport of etoposide glucuronide by MRP3. The calculated K_m and V_{max} of MRP3 for etoposide glucuronide are 11.4 ± 2.6 μM and 138 ± 15 pmol/mg of protein/min, respectively. The calculated V_{max} probably underestimates the actual V_{max}, since in the 5-min assays used, the reaction had already passed the linear phase. Etoposide glucuronide is therefore a good substrate for MRP3.

Analysis of [^{3}H]Etoposide Efflux from MRP3 Cells—Our finding that etoposide glucuronide is a high affinity substrate of MRP3 raised the question of whether the resistance of our *MRP3* transfectants to etoposide could be because of conversion of etoposide into etoposide glucuronide followed by export via MRP3. To test this possibility we analyzed the efflux medium from our cells after they were loaded with [^{3}H]etoposide using an optimized HPLC system. The [^{3}H]etoposide form that was effluxed had a retention time of 16 min, identical to that of a [^{3}H]etoposide standard (Fig. 8). In contrast, etoposide glucuronide had a retention time of 11 min (Fig. 8) and was stable when added to our cells under the experimental conditions used (data not shown). We conclude that the resistance of our MRP3 cells is due to the extrusion of etoposide itself rather than etoposide glucuronide.

DISCUSSION

We have used transfected cells to study the substrate specificity and drug transport mechanism of MRP3. A major complication in studying the contribution of a heterologously introduced transporter to drug resistance of transfected cells is the presence of other endogenous drug transporters with an overlapping substrate specificity. These endogenous transporters can show clonal variation in expression (36). Allen *et al.* (37) demonstrate that even wild type expression levels of the murine Mrp1 or Mdr1a/1b P-glycoproteins have a large effect on the intrinsic resistance of cell lines to some anticancer drugs. Similarly, mice that have a combined deficiency for these transporters are hypersensitive to etoposide and VCR (11, 31, 38). We therefore generated stable clones that overexpress MRP3 in a fibroblast cell line derived from mice that have homozygous disruptions of *Mdr1a/1b* and *Mrp1*. These cells lack three major drug transporters, are hypersensitive to anti-cancer drugs, and are suitable for studying the contribution of an exogenously introduced transporter to drug resistance. These cells are not devoid of other known transporters, however. We detect low amounts of Mrp5 protein and of *Bcrp* (*Abcg2*) and *Mrp4* RNAs,[2] but this does not affect the interpretation of our transfection experiments. For vesicular transport studies, we expressed MRP3 in insect cells, which have a low endogenous transport capacity for the drugs of interest.

Overexpression of MRP3 in our murine fibroblast cells only resulted in high resistance to etoposide and teniposide (RF ~8 and ~5, respectively). The etoposide resistance was the result of reduced accumulation (Table II) because of enhanced efflux (Fig. 4). In a comparable study of two clones of MRP3-transfected HEK293 cells, resistance to VCR (RF 2.2 and 1.5) was reported (25). This low resistance raises the question whether the VCR resistance of these cells could be because of clonal variation in the expression of endogenous transporters, *e.g.* MRP1 or P-glycoprotein. The cells used in our study, lacking Mrp1 and Mdr1a/1b P-glycoproteins, have the highest reported resistance factors for etoposide as a result of MRP3 overproduction. Additionally, the intrinsic resistance of these cells to VCR and etoposide, as determined by comparing the IC_{50} values of the parental lines, is 2–4-fold lower than of the HEK293 cells used by Zeng *et al.* (25). We think therefore, that the absence of detectable resistance to doxorubicin, VCR, and cis-

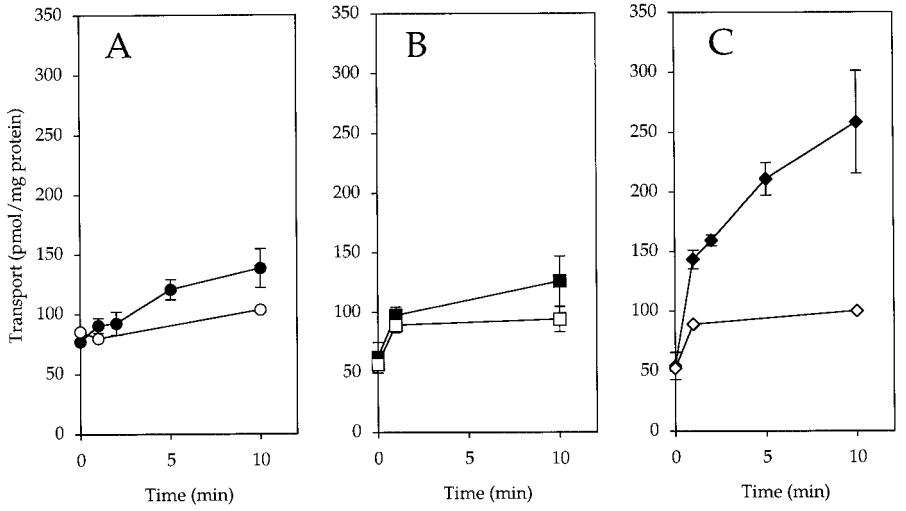

FIG. 5. **Vesicular transport of GSH.** Membrane vesicles prepared from Sf9 cells infected with MRP3 (*A*), wild type (*B*), or MRP1 (*C*) baculovirus were incubated with 0.1 mM [^{3}H]GSH in the presence of 4 mM ATP (*closed symbols*) or AMP (*open symbols*) at 37 °C. To keep the GSH reduced, the reaction was supplemented with 10 mM dithiothreitol. Each *point* and *bar* is the average ± S.E. of an experiment in triplicate.

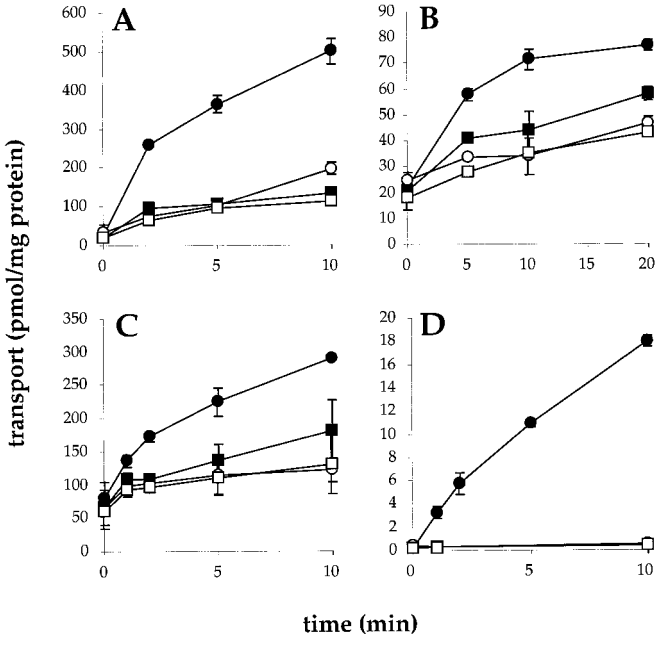

FIG. 6. **Transport of $E_2$17βG, LTC_4, DNP-SG, and etoposide glucuronide by MRP3.** Membrane vesicles prepared from Sf9 cells infected with MRP3 (*circles*) or wild type (*squares*) baculovirus were incubated at 37 °C with 10 μM [^{3}H]$E_2$17βG (*A*), 50 nM [^{3}H]LTC_4 (*B*), 3 μM [^{3}H]DNP-SG (*C*), or 82.5 nM [^{3}H]etoposide glucuronide (*D*) in the presence of 4 mM ATP (*closed symbols*) or AMP (*open symbols*). Each *point* and *bar* is the average ± S.E. of an experiment in triplicate. Note that in *D*, the uptake values for wild type cells coincide with the uptake values for *MRP3* transfectants in the presence of AMP.

TABLE III
Effect of various compounds on ATP-dependent $E_2$17βG transport by human MRP3

Membrane vesicles prepared from Sf9 cells infected with baculovirus-containing MRP3 cDNA were incubated with 0.1 μM [^{3}H]$E_2$17βG at 37 °C. ATP-dependent transport was calculated by subtracting values in the presence of 4 mM AMP from those in the presence of 4 mM ATP. Each value is the average ± S.E. of an experiment done in triplicate, expressed as the percentage of transport in the absence of inhibitor.

Inhibitor	Concentration	ATP-dependent transport
	μM	*% of control without inhibitor*
Sulfinpyrazone	500	67 ± 11
	1000	40 ± 8
Probenecid	250	84 ± 18
	500	59 ± 11
	1000	41 ± 10
Indomethacin	50	92 ± 10
	100	54 ± 6
	250	23 ± 15
Ibuprofen	500	106 ± 0
	1000	123 ± 0
	2000	114 ± 0
Methotrexate	10	114 ± 32
	100	92 ± 12
	1000	54 ± 15
Etoposide	1	91 ± 7
	10	65 ± 10
	100	44 ± 4

platin in these cells indicates that these drugs do not belong to the substrate spectrum of MRP3 or are not pumped at rates that result in detectable drug resistance.

Our results shed new light on the mechanism of etoposide transport by MRP3. Under physiological conditions, etoposide is predominantly present as an electroneutral compound. Efflux of neutral or basic drugs from cells by MRP1 requires GSH (39, 40), and *Vinca* alkaloids are transported by MRP1 and MRP2 together with GSH (28, 29). Etoposide transport by Mrp1 was addressed by Rappa *et al.* (40) using embryonic stem cells that have a homozygous disruption of *Mrp1*. Wild type embryonic stem cells accumulate 2-fold less etoposide than their *Mrp1* (−/−) counterparts. The decreased accumulation of etoposide in these cells was completely reversed by BSO at concentrations similar to the ones used in the present study. Additionally, etoposide strongly stimulated GSH efflux from wild type cells but not from cells devoid of Mrp1. Taken together, these results point to co-transport of GSH with etoposide by Mrp1. In contrast, pretreatment of MRP3-overproducing cells with BSO, causing a 90% reduction in intracellular GSH (Table I), had no effect on the resistance to etoposide or teniposide. Moreover, basal GSH efflux from MRP3 cells was low and could not be stimulated by incubation with etoposide. Additionally, whereas MRP1-enriched membrane vesicles showed significant ATP-dependent uptake of GSH (Fig. 5*C*), MRP3-enriched vesicles did not (Fig. 5, *A* and *B*). Finally, including GSH in the reaction did not stimulate the inhibition of $E_2$17βG vesicular transport by etoposide. It is therefore evident that etoposide transport by MRP3 does not require co-transport with GSH, as implied for MRP1.

This raised the possibility that MRP3 transports a metabolite of etoposide, *e.g.* etoposide glucuronide, a major metabolite of etoposide *in vivo* (35) and a substrate of MRP1 (6, 30). We tested whether this compound is also a substrate for MRP3. Indeed, we found substantial vesicular transport of etoposide glucuronide in MRP3-containing vesicles (Fig. 6*D* and 7), comparable with the rates of transport observed with $E_2$17βG.

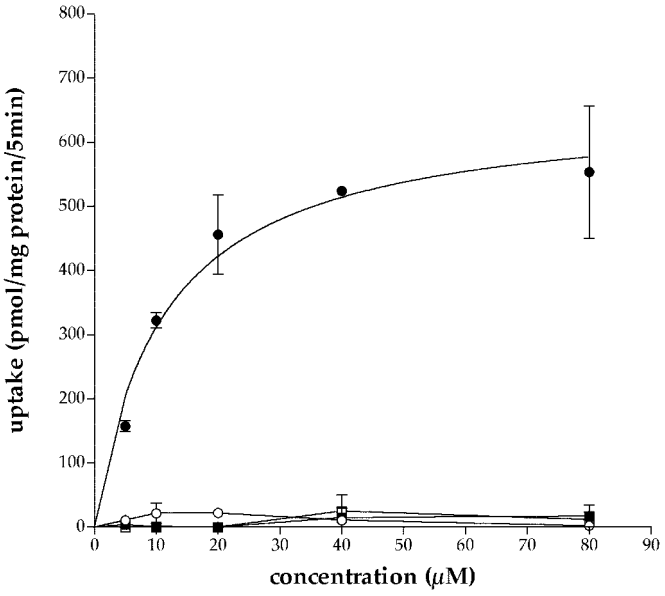

FIG. 7. **Concentration dependence of etoposide glucuronide transport by MRP3.** Membrane vesicles prepared from Sf9 cells infected with MRP3 (*circles*) or wild type (*squares*) baculovirus were incubated for 5 min at 37 °C with ^{3}H-labeled (170 nM) and unlabeled etoposide glucuronide to the final concentration shown in the figure in the presence of 4 mM ATP (*closed symbols*) or AMP (*open symbols*). Each *point* and *bar* is the average ± S.E. of an experiment in triplicate.

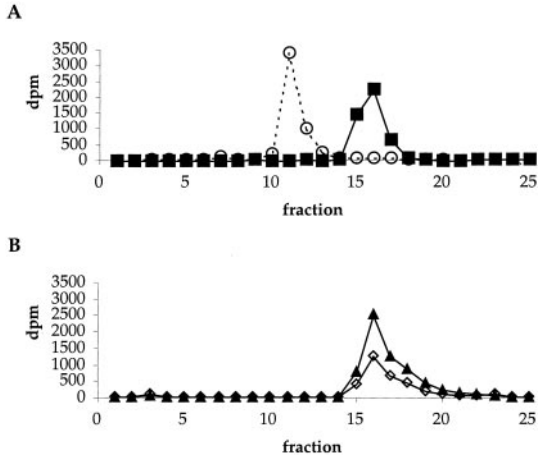

FIG. 8. **HPLC analysis of efflux medium from MRP3 cells.** Medium collected in etoposide efflux assays was analyzed by HPLC as described in materials and methods. *A*, standards of etoposide (■) and etoposide glucuronide (○). *B*, efflux medium from control, V1 (◇) and MRP3 38 (▲) clones.

However, we did not find excretion of any etoposide glucuronide from our etoposide-resistant MRP3 cells (Fig. 8). Presumably, these cells lack the UDP-glucuronosyltransferase required to conjugate etoposide to glucuronate.

The results presented here argue against the two most plausible explanations for etoposide transport mediated by MRP3, co-transport with GSH and transport of a major metabolite. It remains possible that MRP3 co-transports etoposide with another intracellular organic anion present at sufficiently high concentrations, such as 2-oxoglutarate. We prefer the hypothesis, however, that MRP3 recognizes etoposide itself. This would explain the inhibition of vesicular transport by etoposide (Table III). It may also offer a clue as to why MRP3 confers high resistance to etoposide and teniposide but not to podophyllotoxin. The major structural difference between the latter and its two semi-synthetic drug derivatives is the presence of a large glycosidic moiety in the derivatives. Interestingly, plants metabolize many endogenous and exogenous compounds by formation of a glycoside derivative. An MRP-like activity specific for glycosides has been recently described in barley vacuoles for the herbicide hydroxyprimisulfuron glucoside (41). A more trivial explanation for the difference between etoposide and podophyllotoxin could be a difference in influx rate into the cell. Podophyllotoxin is more hydrophobic than etoposide, and it might enter the cell too fast to allow effective defense by a pump.

Our results show that MRP3 could play a role in etoposide and teniposide resistance of cancer cells but argue against a substantial contribution of MRP3 to multidrug resistance comparable with that of MRP1 or P-glycoprotein. The high affinity of MRP3 for the glucuronosyl conjugate of a drug like etoposide suggests, however, that MRP3 could be important in the disposal of toxic compounds inactivated by glucuronidation, a major detoxification pathway in mammals (42). In addition, MRP3 can transport bile salts (23, 24),[2] and it could play a role in the enterohepatic circulation of these salts. The Mrp3 knockout mice recently generated in our laboratory should allow us to test these ideas.

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