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

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Running title:

Drug transport by MRP3
Summary

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 kidney 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, 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 C₄, dinitrophenyl S-glutathione, but not glutathione itself, and of 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.
Introduction

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) family, typified by the 190 kDa MRP1 (3), and of BCRP (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 C4 transporter (LTC4) (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 upregulated 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) established 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 tauroliothocholate-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).

As MRP1 has a broad substrate specificity for anti-cancer 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 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, though 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 kidney of mice that have a targeted disruption of Mdr1a/b, and Mrp1 (31) and in vesicular transport assays.
Experimental Procedures

Materials

[\textsuperscript{3}H]etoposide (411 or 280 mCi/mmol) was purchased from Moravek (Brea, CA). \[\textsuperscript{3}H\]GSH (50 Ci/mmol), \[\textsuperscript{3}H\]LTC\textsubscript{4} (146 Ci/mmol) and estradiol 17-\(\beta\)-D glucuronide (E\textsubscript{2}17\(\beta\)G) (44 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA). Unlabeled and \[\textsuperscript{3}H\]-labeled 2,4-dinitrophenyl S-glutathione (DNP-SG) were synthesized enzymatically using glycine-2-[\textsuperscript{3}H]GSH (NEN Life Science Products) 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 (St. Louis, MO). Solvents used for HPLC analysis were of HPLC grade.

Cell lines

Fibroblasts derived from the kidney of mice that have a homozygous deletion of Mdr1a/b, and Mrp1 (31) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 100 units/ml penicillin/streptomycin. Cells were grown at 37\(^\circ\)C with 5% CO\textsubscript{2} under humidifying conditions. Cell lines were regularly checked for the absence of mycoplasma infection.

Cloning & transfection of cell lines
A cDNA insert encoding MRP3 was excised from pGEM7-MRP3 (16) using AatII and SphI, blunted and cloned into the EcoRV 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 kidney of mice that lack Mdr1a/b, and Mrp1. As control, the same line was transduced with virus particles that contain no MRP3 cDNA insert. After 48 hours, the cells were split and stable clones were selected with 2µg Puromycin per 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 & immunohistochemistry
Total cell lysates were prepared in a hypotonic lysis buffer (10mM KCl, 1.5mM MgCl2, 10mM Tris-HCl pH 7.4), supplemented by a cocktail of protease inhibitors used at the dilution recommended by the manufacturer (Roche, Mannheim, Germany). 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 M3II-9 (1:250), followed by a rabbit anti-mouse HRP conjugate (1:1000) and visualized with chemiluminescence (ECL, Amersham, UK). Mdr1a/b Pgp, and Mrp1 were detected using the mAbs C219 and MRP-R1, respectively. For immunolocalization of MRP3, cells were grown overnight on glass slides. MRP3 was detected using M3II-9 (1:50) as primary antibody and Alexa 488-labeled goat anti-mouse IgG (1:100) as 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 hours, drug in varying concentrations was added to each well. Incubations were continued for an additional 72 hours. For studying the effect of GSH depletion on drug sensitivity cells were incubated for 24 hours with 50 µM buthionine sulfoximine (BSO) prior to the addition of drug. At the end of the assay, the medium was removed from the plates, and plates were frozen at -80°C for at least 4 hours. 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.


One day prior to the assay, cells were plated at a density of 1x10$^6$/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 hour 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, Meriden, CT). 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.


Cells were plated at a density of 1x10$^6$/well one day prior to assay. The following day, cells were washed twice with pre-warmed PBS and incubated with 1 ml of DMEM 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 hour at 37°C. Following this, the cells were washed twice with cold PBS and efflux was initiated by addition of prewarmed complete DMEM. Samples from the
efflux medium were taken at various time points and measured using liquid scintillation counting.

**GSH assays**

To determine intracellular GSH levels, 2x10^6 cells were plated per well. Medium was refreshed 24 hours later. When the effect of BSO on intracellular GSH was studied, medium containing 50 µM BSO was used. After an additional 24 hours the cells were washed twice with PBS and intracellular GSH 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 1ml of HBS containing 500 µM of acivicin to inhibit γ-glutamyl transferase 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. Following the last sample, the cells were washed twice with ice-cold HBS buffer and intracellular GSH determined as above.

**Synthesis of [3H]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 MgCl₂, 50 mM MOPS pH 7.4, and [³H]etoposide (final concentration 100 µM) in a total volume of 200 µl at
37°C for 2 hours. The reaction was terminated by addition of an equal volume of acetonitrile. After removal of protein, the reaction mix was fractionated by HPLC and fractions containing [3H]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-H]-) and ions at m/z 571.0 ([M+H-glucuronic acid]-) with high intensity. Non-labeled 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 [3H]etoposide and potential metabolites was done as described by Hande et al. with minor modifications (35). In brief, efflux medium was mixed with an equal volume of acetonitrile to precipitate proteins, vortexed for 1 minute, and centrifuged at 10000 rpm for 1 minute. 50 µl of the resulting supernatant were injected on a 3.9 x 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 1ml fractions. Radioactivity in the fractions was measured by liquid scintillation.
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 PMSF, 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 x 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 x g at 4°C for 10 min, the supernatant was centrifuged at 4°C at 100,000 x 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 following the rapid filtration method. Briefly, membrane vesicles containing 20 µg protein were incubated with the indicated concentration of substrate in 50 µl of TS-buffer in the presence of 4 mM ATP or AMP, 10 mM MgCl₂, 10 mM creatine phosphate, and 100 µg creatine kinase per 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 measured by liquid scintillation.
Results

Cloning & 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 kidney of mice that lack Mdr1a/b, and Mrp1. Clones that survived selection with 2 µg puromycin per ml were expanded and analyzed by western blotting for MRP3 overproduction. Two clones, MRP3 #38, and MRP3 #77 were used for further study (figure 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 (figure 2). Using the monoclonal antibody M3II-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 1. High resistance was found only against etoposide and teniposide (figure 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, prior to addition of drugs. Preincubation of the cells with 50 µM BSO (a non-toxic concentration, data not shown) resulted in a drastic decrease in intracellular GSH levels (table 1). This had no effect on the resistance of MRP3 cells to etoposide or teniposide (table 1).

[^H]etoposide accumulation assays

To further investigate the mechanism of MRP3-mediated etoposide drug resistance, we performed steady state drug accumulation experiments. Accumulation of[^H]etoposide reached steady state levels after 1 hour of incubation (data not shown) and therefore all assays were conducted for this duration. Cells were incubated with 2 µM[^H]etoposide at 37°C and the
radioactivity retained intracellularly was measured. The V1 control clone accumulated 4.2 ± 0.4 pmol [³H]etoposide /1x10⁶ cells, while MRP3 #38, and MRP3 #77 showed a substantial decrease in accumulation, accumulating 0.8 ± 0.1, and 1.3 ± 0.3 pmol [³H]etoposide /1x10⁶ cells, respectively (n=10). The results are summarized in table 2. 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 2, 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 of 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.

[³H]etoposide efflux assay

To obtain an estimate of the initial rate of MRP3-mediated etoposide transport we performed drug efflux experiments. Since our MRP3 clones accumulate less etoposide than our control clone, we had to use ATP-depleting conditions to load cells with [³H]etoposide. Under these conditions, clones V1, MRP3 #38, and MRP3 #77 showed similar accumulation of [³H]etoposide (18.9 ± 2.1, 16.3 ± 0.4,
16.7 ± 1.9 pmol/1x10^6 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 (figure 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, #77 were 1.7x
and 1.5x, respectively, higher than that found in the control clone.

Analysis of GSH transport by MRP3

The results in table 1 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 hr (0.8 ± 0.4, 0.6 ± 0.3,
and 0.6 ± 0.4 nmol GSH/mg protein, respectively, n=3) and this was not
significantly increased in the presence of 34µM of etoposide (1.1 ± 0.3, 0.8 ± 0.4,
and 0.7 ± 0.3 nmol GSH/mg protein for clones V1, MRP3 #38, and MRP3 #77,
respectively). In contrast, a clone that overproduces MRP1 (clone MRP1 #2.2,
unpublished) showed a 4-fold higher basal level of GSH efflux than MRP3 cells
and this efflux rate was two-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 total cell lysates of the fibroblast clone MRP3 #38 as determined by densitometry (figure 1). Uptake of GSH into these vesicles (figure 5a) was not higher than into control vesicles (figure 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 (figure 5c). Membranes from mammalian cells transfected with rat Mrp3 or human MRP3 cDNA constructs transport 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$17βG in an ATP-dependent manner (figure 6a) with K$_m$ and V$_{max}$ values of 17.7 ± 2.6 µM and 474 ± 63 pmol/mg 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 wildtype baculovirus (figure 6b,c), in agreement with results obtained by Zeng et al. (24) with mammalian cell vesicles. The ATP-dependent uptake of E$_2$17βG by MRP3-containing vesicles was inhibited by MTX and etoposide as well as by the same inhibitors used in the etoposide accumulation assays (table 3). 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. Figure 6d 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. As 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. Figure 7 shows the concentration-dependent transport of etoposide glucuronide by MRP3. The calculated $K_m$ and $V_{max}$ of MRP3 for etoposide glucuronide are $11.4 \pm 2.6\mu\text{M}$ and $138 \pm 15 \text{ pmol/mg protein/min}$, respectively. The calculated $V_{max}$ probably underestimates the actual $V_{max}$ as in the 5 minute assays used the reaction has already passed the linear phase. Etoposide glucuronide is therefore a good substrate for MRP3.

**Analysis of $[^3\text{H}]$etoposide efflux from MRP3 cells**

Our finding that etoposide glucuronide is a high-affinity substrate of MRP3 raised the question whether the resistance of our MRP3 transfectants to etoposide could be due to 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 [³H]etoposide using an optimized HPLC system. The [³H]etoposide form that was effluxed had a retention time of 16 minutes, identical to that of a [³H]etoposide standard (figure 8). In contrast, etoposide glucuronide had a retention time of 11 minutes (figure 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. demonstrated that even wildtype 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 (37). 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, they 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 (unpublished results), 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 2) due to enhanced efflux (figure 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 due to 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 RFs 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\textsubscript{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 cisplatin 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. using embryonic stem (ES) cells that have a homozygous disruption
of Mrp1 (40). Wildtype ES cells accumulate two-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
wildtype 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 1), had no effect on the resistance to etoposide or teniposide.
Moreover, basal GSH efflux from MRP3 cells is low and cannot be stimulated by
incubation with etoposide. Additionally, whereas MRP1-enriched membrane
vesicles show significant ATP-dependent uptake of GSH (figure 5c), MRP3-
enriched vesicles do not (figure 5a,b). Finally, including GSH in the reaction did
not stimulate the inhibition of E217βG 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 (figure 6d and 7), comparable to the
rates of transport observed with E217βG. However, we did not find excretion of
any etoposide glucuronide from our etoposide-resistant MRP3 cells (figure 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-transport 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 3). 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 to 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) and our unpublished observations) 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.
Acknowledgments

We thank our colleagues Peter Wielinga, Hein te Riele, and Alfred Schinkel (The Netherlands Cancer Institute), and Jan Wijnholds (University of Amsterdam) for critical reading of the manuscript, Lauran Oomen (The Netherlands Cancer Institute) for assistance with the confocal laser scanning microscopy, Danny Burg (University of Leiden) for providing us with rat liver microsomes and synthesizing DNP-SG and Brian Burchell (University of Dundee) for his advice on the synthesis of etoposide glucuronide. The Mdr1a/1b (-/-), Mrp1 (-/-) kidney derived fibroblast cell line was kindly provided by Remco Brinkhuis and Alfred Schinkel (The Netherlands Cancer Institute). T. S. was supported by a postdoctoral fellowship from the Japanese Society for the Promotion of Science and our research was supported by grants (NKI 2001-2474 and 1998-1794) of the Dutch Cancer Society to P.B.
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Footnotes

1 The abbreviations used are: ABC, ATP binding cassette; MTX, methotrexate; VCR, vincristine; GSH, glutathione; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; BSO, buthionine sulfoximine; HBS, Heps-buffered saline solution; ES, Embryonic stem cells; Sf9, *Spodoptera frugiperda*; E217βG, estradiol 17-β-D-glucuronide; LTC4, leukotriene C4; DNP-SG, 2,4-dinitrophenyl S-glutathione.
Legends to figures

Figure 1. Immunoblot analysis of MRP3 expression in selected clones. MRP3 expression in Sf9-MRP3 membranes and in selected transfected fibroblast clones. SF9-MRP3 membranes containing 1-4 µg protein, or 15 or 30 µg of total cellular protein were loaded per lane and MRP3 was detected using mAb M3II-9 (16).

Figure 2. Immunolocalization of MRP3. MRP3 was immunolocalized by primary mAb M3II-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.

Figure 3. Drug resistance of MRP3 cells. 1000 cells per well were plated in triplicate 24 hours prior to addition of drug. Cells were exposed to drug for 72 hours after which cellular growth was quantified. A representative growth inhibition assay for etoposide (A), and teniposide (B). Control, V1 (ï¸), MRP3 #38 (+), and MRP3 #77 (û). Each point represents the average ± SE of a measurement done in triplicate.

Figure 4. Etoposide efflux from MRP3 cells. Cells were incubated with 2.5 µM [³H]etoposide under ATP-depleting conditions for one hour. Subsequently, efflux was initiated into complete medium and samples 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 (○), and MRP3 #38 (+). Each point represents the average ± SE of a measurement done in triplicate.

**Figure 5. Vesicular transport of GSH.** Membrane vesicles prepared from Sf9 cells infected with MRP3 (A), wildtype (B), or MRP1 (C) baculovirus were incubated with 0.1 mM [³H]GSH in the presence of 4 mM ATP (closed symbols) or AMP (open symbols) at 37°C. In order to keep the GSH reduced, the reaction was supplemented with 10mM DTT. Each point and bar is the average ± SE of an experiment in triplicate.

**Figure 6. Transport of E₂₁βG, LTC₄, DNP-SG, and etoposide-glucuronide by MRP3.** Membrane vesicles prepared from Sf9 cells infected with MRP3 (circles) or wildtype (squares) baculovirus were incubated at 37°C with 10 µM [³H]E₂₁βG (A), 50 nM [³H]LTC₄ (B), 3 µM [³H]DNP-SG (C), or 82.5 nM [³H]etoposide glucuronide (D) in the presence of 4 mM ATP (closed symbols) or AMP (open symbols). Each point and bar is the average ± SE of an experiment in triplicate. Note that in figure 6d, the uptake values for wildtype cells coincide with the uptake values for MRP3 transfectants in the presence of AMP.

**Figure 7. Concentration dependence of etoposide glucuronide transport by MRP3.** Membrane vesicles prepared from Sf9 cells infected with MRP3 (circles)
or wildtype (squares) baculovirus were incubated for 5 minutes at 37°C with [3H]-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 ± SE of an experiment in triplicate.

Figure 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 (Á). (B) Efflux medium from Control, V1 (ô), and MRP3 #38 (û) clones.
Table 1

Table 1. **Drug resistance phenotype of MRP3 cells.** 1000 cells were plated in triplicate 24 hours prior to addition of drug. Cells were grown for an additional 72 hours in the presence of a varying concentration of drug after which cellular growth was measured and the concentration (±SD) that inhibits cells growth by 50% determined (IC$_{50}$).

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

$^a$ Relative resistance factor (RF) 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 hours with 50µM BSO prior to addition of drug. 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 (nmoles GSH/mg protein) for clones V1, MRP3 #38, and MRP3 #77 respectively.

$^d$ N.D. not determined.
**Table 2.**

**Steady state etoposide accumulation in MRP3 overproducing cells.**

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>V1</th>
<th>MRP3 #38</th>
<th>MRP3 #77</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>100 ± 0</td>
<td>18 ± 3</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>Benzbromarone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 µM</td>
<td>105 ± 8</td>
<td>19 ± 3</td>
<td>26 ± 11</td>
</tr>
<tr>
<td>100 µM</td>
<td>110 ± 4</td>
<td>28 ± 3</td>
<td>37 ± 8</td>
</tr>
<tr>
<td>250 µM</td>
<td>97 ± 1</td>
<td>73 ± 7</td>
<td>67 ± 11</td>
</tr>
<tr>
<td>Indomethacin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µM</td>
<td>88 ± 6</td>
<td>26 ± 4</td>
<td>31 ± 9</td>
</tr>
<tr>
<td>250 µM</td>
<td>84 ± 2</td>
<td>53 ± 3</td>
<td>56 ± 6</td>
</tr>
<tr>
<td>500 µM</td>
<td>76 ± 1</td>
<td>67 ± 2</td>
<td>64 ± 6</td>
</tr>
<tr>
<td>Probenecid</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0.5 mM</td>
<td>89 ± 19</td>
<td>30 ± 3</td>
<td>39 ± 8</td>
</tr>
<tr>
<td>1 mM</td>
<td>91 ± 14</td>
<td>41 ± 4</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>5 mM</td>
<td>76 ± 9</td>
<td>59 ± 5</td>
<td>58 ± 5</td>
</tr>
<tr>
<td>Sulfinpyrazone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 mM</td>
<td>100 ± 1</td>
<td>27 ± 14</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>1 mM</td>
<td>101 ± 2</td>
<td>50 ± 25</td>
<td>65 ± 0</td>
</tr>
<tr>
<td>2 mM</td>
<td>92 ± 1</td>
<td>83 ± 4</td>
<td>77 ± 1</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td></td>
<td></td>
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<tr>
<td>1 µM</td>
<td>101 ± 5</td>
<td>19 ± 1</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>10 µM</td>
<td>105 ± 9</td>
<td>17 ± 2</td>
<td>22 ± 5</td>
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<tr>
<td>100 µM</td>
<td>99 ± 9</td>
<td>16 ± 1</td>
<td>23 ± 4</td>
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</tbody>
</table>
Table 3. Effect of various compounds on ATP-dependent E$_2$17βG transport by human MRP3. Membrane vesicles prepared from Sf9 cells infected with baculovirus containing MRP3 cDNA were incubated with 0.1 µM [³H]E$_2$17βG at 37°C. ATP-dependent transport was calculated by subtracting values in the presence of 4 mM AMP from those of in the presence of 4 mM ATP. Each value is the average ± SE of an experiment done in triplicate, expressed as percentage of transport in the absence of inhibitor.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>concentration (µM)</th>
<th>ATP-dependent transport (% of control without inhibitor)</th>
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<tr>
<td>sulfinpyrazone</td>
<td>500</td>
<td>67 ± 11</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>40 ± 8</td>
</tr>
<tr>
<td>probenecid</td>
<td>250</td>
<td>84 ± 18</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>59 ± 11</td>
</tr>
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<td></td>
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<td>41 ± 10</td>
</tr>
<tr>
<td>indomethacin</td>
<td>50</td>
<td>92 ± 10</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>54 ± 6</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>23 ± 15</td>
</tr>
<tr>
<td>ibuprofen</td>
<td>500</td>
<td>106 ± 0</td>
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<tr>
<td></td>
<td>1000</td>
<td>123 ± 0</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>114 ± 0</td>
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<tr>
<td>methotrexate</td>
<td>10</td>
<td>114 ± 32</td>
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<tr>
<td></td>
<td>100</td>
<td>92 ± 12</td>
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<tr>
<td></td>
<td>1000</td>
<td>54 ± 15</td>
</tr>
<tr>
<td>etoposide</td>
<td>1</td>
<td>91 ± 7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>65 ± 10</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>44 ± 4</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2.
Figure 3.
Figure 4.
Figure 5.

A

B

C

Transport (pmol/mg protein)

Time (min)
Figure 6.
Figure 7.
Figure 8.
Characterization of drug transport by the human multidrug resistant protein 3 (ABCC3)
Noam Zelcer, Tohru Saeki, Glen Reid, Jos H. Beijnen and Piet Borst

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