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Characterization of the Transport of Nucleoside Analog Drugs by the Human Multidrug Resistance Proteins MRP4 and MRP5

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ABSTRACT

The human multidrug resistance proteins MRP4 and MRP5 are organic anion transporters that have the unusual ability to transport cyclic nucleotides and some nucleoside monophosphate analogs. Base and nucleoside analogs used in the chemotherapy of cancer and viral infections are potential substrates. To assess the possible contribution of MRP4 and MRP5 to resistance against these drugs, we have investigated the transport mediated by MRP4 and MRP5. In cytotoxicity assays, MRP4 conferred resistance to the antiviral agent 9-(2-phosphonomethoxyethyl)adenine (PMEA) and high-performance liquid chromatography analysis showed that, like MRP5, MRP4 transported PMEA in an unmodified form. MRP4 also mediated substantial resistance against other acyclic nucleoside phosphonates, whereas MRP5 did not. Apart from low-level MRP4-mediated cladribine resistance, the cytotoxicity of clinically used anticancer nucleosides was not influenced by overexpression of MRP4 or MRP5. In contrast, MRP5 mediated efflux of the pyrimidine-based antiviral 2′,3′-dideoxynucleoside 2′,3′-didehydro-2′,3′-dideoxythymidine 5′-monophosphate (d4TMP) and its phosphoramidate derivative alaninyl-d4TMP from cells loaded with the 2′,3′-didehydro-2′,3′-dideoxythymidine prodrugs cyclosaligenyl-d4TMP and aryloxyphosphoramidate d4TMP (So324), respectively. Moreover, only inside-out membrane vesicles derived from MRP5-overexpressing cells accumulated alaninyl-d4TMP. Cellular efflux and vesicular uptake studies were carried out to further compare transport mediated by MRP4 and MRP5 and showed that dipyridamole, dilazep, nitrobenzyl mercaptopurine riboside, sildenafil, trequinsin and MK571 inhibited MRP4 more than MRP5, whereas cyclic nucleotides and monophosphorylated nucleoside analogs were equally poor inhibitors of both pumps. These results strongly suggest that the affinity of MRP4 and MRP5 for nucleotide-based substrates is low.

The metabolism and flux of nucleosides and nucleoside analogs is a complex process involving a large array of intracellular metabolizing enzymes as well as several families of membrane transport proteins. The transport of nucleoside analogs into and out of cells and their activation by cellular kinases has received much attention because of the influence of these processes on the efficacy of nucleoside analog-based anticancer and antiviral therapies. Although the release of nucleoside monophosphates from cells is documented, the transport proteins involved and their potential effect on nucleoside analog therapies remained unknown until two of the newer members of the multidrug resistance protein (MRP) family of ATP-dependent efflux pumps, MRP4 and MRP5, were shown to transport nucleoside monophosphates (Schuetz et al., 1999; Lee et al., 2000; Wijnholds et al., 2000; Chen et al., 2001). Continuous selection of the human T lymphoblast CEM cell line with the antiviral drug 9-(2-phosphonomethoxyethyl)adenine (PMEA) led to an amplification of the *MRP4* gene with a concomitant decrease in PMEA accumulation (Schuetz et al., 1999). We showed that MRP5-transfected HEK293 cells were resistant against both 6-mercaptopurine, a nucleobase analog drug used in the treatment

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ABBREVIATIONS: MRP, multidrug resistance protein; bis-POM-PMEA, bis(pivaloyloxymethyl)-9-(2-phosphonomethoxyethyl)adenine; cGMP, guanosine 3′,5′-cyclic monophosphate; cPr-PMEDAP, cyclopropyl-PMEDAP; cycloSAL-d4TMP, cyclosaligenyl-d4T-5′-monophosphate; ddC, 2′,3′-dideoxycytidine; d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; d4TMP, 2′,3′-didehydro-2′,3′-dideoxythymidine 5′-monophosphate; DMEM, Dulbecco's modified Eagle's medium; $E_2 17\beta G$, estradiol glucuronide; HEK, human embryonic kidney; HPLC, high-performance liquid chromatography; MK571, 3-[[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoylethylsulfanyl)methylsulfanyl] propionic acid; PMEA, 9-(2-phosphonomethoxyethyl)adenine; PMEDAP, 9-(2-phosphonomethoxyethyl)-2,6-diaminopurine; PMEG, 9-(2-phosphonomethoxyethyl)guanine; PDE, phosphodiesterase; So324, aryloxyalaninylphosphoramidate of d4T-5′-monophosphate (d4TMP).

of acute lymphoblastic leukemia (Wijnholds et al., 1999), and PMEA (Wijnholds et al., 2000). Further analysis indicated that the resistance to PMEA was caused by the efflux of PMEA itself, and resistance to 6-mercaptopurine was caused by the increased efflux of thiopurine nucleoside monophosphates (Wijnholds et al., 2000; Wielinga et al., 2002). Several groups then reported that MRP4 and MRP5 transport the second messengers cyclic GMP and cyclic AMP (Jedlitschky et al., 2000; Chen et al., 2001; Lai and Tan, 2002; van Aubel et al., 2002). Both pumps transported cGMP with high affinity; MRP4 also exhibited high-affinity cAMP transport (Jedlitschky et al., 2000; Chen et al., 2001). Most recently, MRP4 was shown to efflux the monophosphorylated form of the antiviral drug ganciclovir, an antiviral agent used clinically for the treatment of cytomegalovirus infections (Adachi et al., 2002).

The ability of MRP4 and MRP5 to transport monophosphorylated forms of several nucleoside analog drugs could affect both anticancer and antiviral therapies involving these classes of drug. In addition to the thiopurines, deoxynucleoside analogs such as cytarabine and cladribine are also used in the treatment of a variety of leukemias, and gemcitabine is used in the treatment of a number of solid tumors (Galmarini et al., 2001). Deoxynucleoside analogs are taken up by nucleoside transporters and, like thiopurines, are converted to the active triphosphate by a succession of cellular kinases before incorporation into nucleic acids. Nucleoside analogs are also integral components of antiviral therapy; for example, the 2′,3′-dideoxynucleoside drugs didanosine (2′,3′-dideoxyinosine), zalcitabine [2′,3′-dideoxycytidine (ddC)], and stavudine [2′,3′-didehydro-3′deoxythymidine (d4T)] are all used clinically to treat HIV infections (De Clercq, 2001). Although many compounds of this class of nucleoside analog inhibit viral replication, they are often of limited use because they are poorly activated to the monophosphate form by cellular nucleoside kinases (Starnes and Cheng, 1987; Balzarini et al., 1989; Johnson and Fridland, 1989). The kinase requirement can be circumvented via direct intracellular delivery of dideoxynucleosides in their monophosphate forms (Balzarini et al., 1996b, 2000), for instance by cell-permeable phosphoramidate triester and cyclosaligenyl dideoxynucleotide prodrugs, exemplified by the aryloxyalaninyl phosphoramidate of d4TMP (So324) and cyclosaligenyl-d4T-5′-monophosphate (cycloSal-d4TMP), respectively (see Fig. 1). Likewise, acyclic nucleoside phosphonates are preformed nucleoside monophosphate analogs that do not require activation by nucleoside kinases.

Because both MRP4 and MRP5 have previously been shown to transport nucleoside monophosphates, this is a potential mechanism for reduced efficacy of therapies involving nucleoside analogs. We have investigated this possibility by comparing the resistance profile and transport properties of MRP4- or MRP5-overexpressing HEK293 cells exposed to a range of both clinically used and experimental nucleoside analog drugs. We find that although both transporters efflux a number of nucleoside analogs in the HEK293 background, MRP4 mediates substantial resistance to a wider variety of acyclic nucleoside phosphonates than MRP5, whereas only MRP5 transports the monophosphorylated form of the dideoxynucleoside d4T and its prodrugs.

Materials and Methods

Materials. [^{3}H]PMEA, [^{3}H]PMEG, and [8-^{3}H]bis-POM-PMEA were purchased from Moravek Biochemicals (Brea, CA), and [^{3}H]estradiol glucuronide was from PerkinElmer Life Sciences (Boston, MA). PMEA and bis-POM-PMEA were kindly provided by N. Bischofberger (Gilead Sciences, Foster City, CA). PMEDAP, cPr-PMEDAP, and 1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine were a kind gift from A. Holy (Prague, Czech Republic, and Gilead Sciences). The monophosphates of gemcitabine, 1-β-D-arabinofuranosylcytosine and ddC, were synthesized by C. Meier (Hamburg, Germany) and purified by HPLC. Fludarabine and [^{3}H]fludara were kind gifts from Dr. J. Gay (Schering, Berlin, Germany). Cladribine was obtained from Janssen-Cilag (The Netherlands), gemcitabine was from Eli Lilly (The Netherlands), MK571 was from Biomol (Plymouth Meeting, PA), cytarabine was from Pharmacia (Woerden, the Netherlands), 5-fluorouracil was from TEVA (Mijdrecht, the Netherlands), and sildenafil was from Pfizer (the Netherlands). Azidothymidine, d4T, and abacavir were obtained from the Pharmacy Department of The Netherlands Cancer Institute. [^{3}H]Alaninyl-dTMP was synthesized from [^{3}H]So324 by incubation with pig liver carboxylesterase (Sigma, St. Louis, MO) as described previously (Balzarini et al., 1996b). The syntheses of So324 and cycloSal-d4TMP have been described previously (Balzarini et al., 1996a, 1999; McGuigan et al., 1996; Meier et al., 1998). All other compounds were purchased from Sigma.

Cell Lines. HEK293/5I and HEK293/5E cells transduced with MRP5 and the MRP4-overexpressing HEK293/4.3 and HEK293/4.63 cells were described previously (Wijnholds et al., 2000; Wielinga et al., 2002). Analyzing serial dilutions of cell lysates on Western blot by densitometry shows approximately 25 and 75 times more MRP4 in the HEK293/4.3 and HEK293/4.63 cells, respectively, and 80 times more MRP5 in the HEK293/5I cells (data not shown). The HEK293/MRP4.59 cells were derived from the same transduction but do not show MRP4 overexpression; HEK293 parental cells and all transfectants were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and 100 units of penicillin/streptomycin per milliliter (Invitrogen), at 37°C under 5% CO_2 humidified air. The cells were routinely checked for the absence of mycoplasma infection and for MRP4 and MRP5 expression levels.

Cytotoxicity. Relative growth of the cells in the presence of the different compounds was tested as described previously (Wijnholds et al., 2000). In short, cells were plated in triplicate in 96-well plates

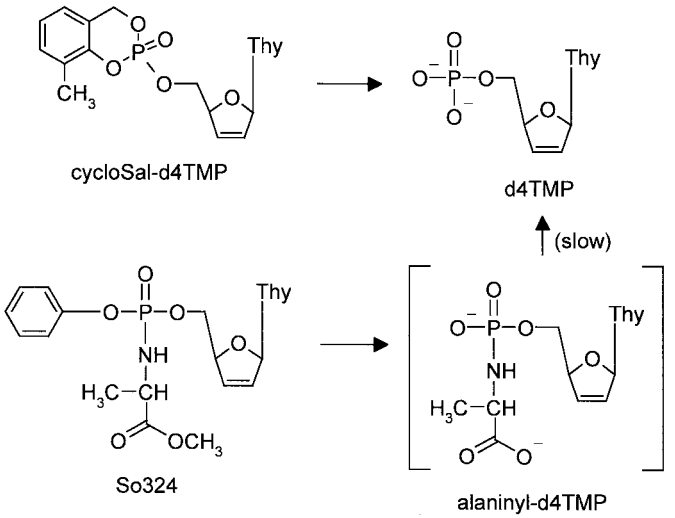


Fig. 1. Molecular structures of d4T prodrugs. The prodrugs So324 and cycloSal-d4TMP are taken up passively into the cell, after which hydrolysis releases d4TMP from cycloSal-d4TMP and alaninyl-d4TMP from So324. The phosphoramidate alaninyl-d4TMP is then slowly converted to d4TMP by cellular phosphoramidases. Thy, thymine. Adapted from Saboulard et al. (1999) and Balzarini et al. (2000).

(1500 cells/well); on the following day, drugs were added at the appropriate dilutions. On day 5, the cell growth in the wells was determined using CyQuant cell proliferation assay kit (Molecular Probes), and fluorescence in each well was determined with a CytoFluor 4000 plate reader (Applied Biosystems, Foster City, CA). The relative resistance was calculated as the ratio of the concentration of drug inhibiting growth by 50% (IC_{50}) in the transfected cell line divided by that in the parental cell line.

Cellular Transport. Cells (2×10^6 per well) were seeded in poly(D-lysine)-coated six-well plates and grown overnight. For the transport experiments, cells were loaded with 1 μM [^{3}H]bis-POM-PMEA, 0.27 μM [^{3}H]cycloSal-d4TMP, or 2.5 μM [^{3}H]So324 for 2 h at 37°C, under ATP-depletion conditions (DMEM without glucose, supplemented with 10% dialyzed fetal calf serum, 10 mM sodium azide, and 10 mM 2-deoxyglucose), plus compounds at the indicated concentrations for the inhibition studies. After loading, the cells were washed rapidly with ice-cold phosphate-buffered saline, followed by addition of prewarmed DMEM. The time-dependent efflux was measured at 37°C by drawing samples over time. Radioactivity in the samples was determined by liquid scintillation counting. Initial efflux rates were determined by linear regression analysis of the linear component of efflux [0–30 min for efflux from So324-loaded cells, 0–15 min for cycloSAL-d4TMP–loaded cells, and 0–60 min for bis-POM-PMEA-loaded cells].

HPLC. Cells were loaded and allowed to efflux as described above, with the exception that after loading and washing, Hanks' buffered salt solution (Invitrogen) was added for the efflux determinations. Metabolites effluxed from cells preloaded with [^{3}H]bis-POM-PMEA, [^{3}H]cycloSal-d4TMP, or [^{3}H]So324 were determined as described previously (Balzarini et al., 1996a, 1998; Hatse et al., 1998).

Preparation of Membrane Vesicles. For the preparation of membrane vesicles, HEK293, HEK293/5I, and HEK293/4.63 cells were grown as described above. 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 aprotinin/ml, 5 μg/ml leupeptin, and 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 25 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 uptake of various substrates into membrane vesicles was studied after the rapid filtration method as described previously (Zelcer et al., 2001). MRP4-mediated transport of [^{3}H]estradiol 17β-glucuronide was measured for 10 min and MRP5-mediated transport of [^{3}H]alaninyl-d4TMP was measured for 15 min. For inhibition studies, uptake in the absence and presence of inhibitors was compared.

Results

Resistance to Nucleoside Analogs Mediated by MRP4 and MRP5. We have tested the effect of *MRP4* and *MRP5* expression in HEK293 cells on the cytotoxicity of a range of base, nucleoside, and nucleotide analogs. The results are presented in Table 1, with some previously published data to provide a complete overview. In cells overproducing MRP4, we see substantial resistance for the acyclic nucleoside phosphonates PMEA [confirming the results of Lee et al. (2000) and Lai and Tan (2002)] and its precursor bis-POM-PMEA and PMEDAP and its derivative cPr-PMEDAP. Resistance was proportional to MRP4 levels, because the HEK293/4.63 cells contain more MRP4 than the HEK293/4.3 cells (Wielinga et al., 2002). The MRP4 cells also showed a lower level of resistance to another acyclic nucleoside phosphonate, PMEG. In contrast, PMEA and bis-POM-PMEA were the only phosphonates significantly affected by MRP5 (this study and Wijnholds et al., 2000). MRP4 and MRP5 also mediated resistance to the purine-based carbocyclic nucleoside analog abacavir (2- and 1.9-fold, respectively).

The overexpression of MRP4 or MRP5 did not alter the toxicity of the clinically important anticancer drugs cytarabine, gemcitabine, fludarabine, or 5-fluorouracil (Table 1). In addition to the previously reported thiopurine resistance (Lee et al., 2000; Wijnholds et al., 2000; Wielinga et al., 2002) replicated here, we only found low-level MRP4-mediated resistance against the anticancer nucleoside analog cladribine (2.2-fold).

TABLE 1

Drug resistance phenotype of HEK293 cells expressing MRP4 or MRP5

No cytotoxicity was observed with So324, azidothymidine, d4T, 2′,3′-dideoxyadenosine, ddC, or 2′,3′-dideoxyinosine. Drugs were tested over a concentration range corresponding to at least 16-fold higher and lower than the IC_{50}. The values in parentheses are the number of determinations

	IC_{50}	Resistance Factor		
	HEK293	HEK293/5I (MRP5)	HEK293/4.3 (MRP4)	HEK293/4.63 (MRP4)
		μM		
Antiviral drugs				
PMEDAP	64 ± 3 (4)	1.7 ± 0.2 (3)	4.8 ± 0.7 (3)	9.6 ± 0.9 (4)
cPr-PMEDAP	2 ± 0.2 (6)	1.4 ± 0.1 (4)	9.6 ± 1.1 (4)	16.6 ± 2.1 (5)
PMEG	1.5 ± 0.1 (4)	1.2 ± 0.2 (3)	2.4 ± 0.3 (4)	3.5 ± 0.3 (3)
PMEA[a]	84.1 ± 15 (6)	2.9 ± 0.3 (10)	3.6 ± 0.3 (6)	7.3 ± 0.2 (2)
bis-POM-PMEA[a]	1.7 ± 0.5 (6)	3.4 ± 0.5 (4)	3.3 ± 0.2 (6)	8.5 ± 0.1 (2)
(*S*)-HPMPC	136 ± 80 (2)	1.2 ± 0.5 (2)	1.2 ± 0.5 (2)	1.2 ± 0.5 (2)
Abacavir	244 ± 86 (4)	1.9 ± 0.4 (4)[b]	2 ± 0.2 (4)	2 ± 0.2 (3)
Anticancer drugs				
Cladribine	2.0 ± 0.4 (6)	1.5 ± 0.3 (4)	1.7 ± 0.3 (5)	2.2 ± 0.4 (3)
Gemcitabine	0.04 (5)	1.0 ± 0.2 (3)	1.1 ± 0.1 (5)	1 ± 0.1 (4)
Cytarabine	1.1 ± 0.3 (5)	1.0 ± 0.2 (5)	1.3 ± 0.3 (5)	1.2 ± 0.3 (5)
Fludarabine	67 ± 10 (4)	1.1 ± 0.2 (4)	1.1 ± 0.2 (4)	1.3 ± 0.4 (2)
5-Fluorouracil	18 ± 2 (2)	1 ± 0.2 (2)	1 ± 0.2 (2)	1.2 (1)
Thioguanine[a]	1 ± 0.2 (4)	2.7 ± 0.1 (2)	2.9 ± 0.6 (3)	4.1 ± 0.1 (3)
6-Mercaptopurine[a]	5.2 ± 0.9 (4)	3.0 ± 0.3 (4)	2.7 ± 0.4 (3)	5.6 ± 2.4 (3)

[a] Similar data were obtained by Wijnholds et al. (2000) and Wielinga et al. (2002).
[b] only value equally significant in a Student's t test, $P < 0.05$ for all RF values ≥ 2.
(*S*)-HPMPC, 1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine.

Efflux from Cells Loaded with d4TMP Prodrugs. In HEK293 cells, we saw no toxicity with the 2′,3′-dideoxynucleosides ddC, 2′,3′-dideoxyinosine, azidothymidine, d4T, or the d4TMP prodrug So324 at concentrations of up to 1 mM, the highest concentrations possible in the presence of less than 1% dimethyl sulfoxide (data not shown). This was not caused by sluggish uptake, because HEK293 cells were found to express the nucleoside transporters hENT1 and hENT2 and accumulate 2′,3′-dideoxycytidine (data not shown). However, it is known that many of these analogs are poor substrates of nucleoside kinases; as such, they are slowly activated to the monophosphate form (Starnes and Cheng, 1987; Balzarini et al., 1989; Johnson and Fridland, 1989). To circumvent the need for a nucleoside kinase, membrane-permeable aryloxyalaninylphosphoramidate and cyclosaligenyl prodrugs of nucleoside 5′-monophosphate derivatives have been developed that result in direct intracellular delivery of free nucleoside monophosphates (reviewed in Meier, 1998). These drugs, however, might be susceptible to efflux by MRP4 or MRP5.

We have investigated this with radiolabeled So324 and cycloSal-d4TMP, cell-permeable prodrugs of the pyrimidine nucleotide d4TMP (see Fig. 1). Once inside cells, the prodrugs are hydrolyzed, releasing alaninyl-d4TMP from So324 and d4TMP from cycloSal-d4TMP. The alaninyl-d4TMP can be further converted into d4TMP upon phosphoramidase-catalyzed cleavage (Saboulard et al., 1999). To determine the influence of MRP4 and MRP5 on intracellular levels of the active drug, cells were loaded with 2.5 μM [^{3}H]So324 or 0.27 μM [^{3}H]cycloSal-d4TMP under ATP-depleting conditions, after which efflux was determined (Fig. 2). Efflux of radiolabel from So324-loaded HEK293/5I (MRP5) cells was rapid, with an initial rate approximately 4-fold higher than in the parental cells, resulting in efflux of almost 80% of the loaded radiolabel within the first hour (Fig. 2A). In contrast, efflux from HEK293/4.3 (MRP4) was equivalent to the parental cells (Fig. 2A). CycloSal-d4TMP–loaded HEK293/5I cells effluxed radioactive compounds faster than HEK293 cells, especially during the first 15 min, although both cell lines effluxed almost all preloaded radiolabel within 2 h (Fig. 2B). The differences in efflux were not a result of differential loading, because the cell lines accumulated equivalent amounts during the preloading period.

To determine the metabolite(s) of cycloSal-d4TMP and So324 transported by MRP5, we analyzed the radiolabel in the efflux medium and the cellular contents by HPLC. Analysis of the efflux media from [^{3}H]cycloSal-d4TMP–loaded parental HEK293 and HEK293/5I (MRP5) cells showed considerable release of both the prodrug and d4TMP from both cell lines after 30 min (Fig. 3A). The efflux of [^{3}H]d4TMP from the HEK293/5I cells (Fig. 3A, ■) was 1.5-fold greater than from the HEK293 parental cells (Fig. 3A, □), strongly suggesting that d4TMP was transported by MRP5. Some intracellular [^{3}H]d4TMP was detected in the HEK293/5I cells (Fig. 3B), but this was 5-fold lower than in the parental cells, where it was the major metabolite found. These data support the conclusion that MRP5 mediates d4TMP efflux, but the extensive efflux of d4TMP from the parental cells suggests that unidentified endogenous transporters also efflux this metabolite. In addition, low levels of phosphorylated forms of [^{3}H]d4TMP, namely d4TDP and d4TTP, were found only in the parental cells. Similar results were obtained with another MRP5 transfectant, HEK293/5E (data not shown). An additional intracellular metabolite accounting for approximately 10% of the radioactivity was detected in the parental cells, but the nature of this metabolite could not be identified with the HPLC system employed.

Figure 3, C and D, show the levels of extracellular and intracellular metabolites after 30-min efflux from So324-loaded parental HEK293 and HEK293/5I (MRP5) cells. Once inside the cell, So324 is rapidly hydrolyzed by intracellular esterases to the d4T prodrug alaninyl-d4TMP, which is relatively stable and only slowly converted to d4TMP (Saboulard et al., 1999). Figure 3 shows that alaninyl-d4TMP is the major metabolite formed in both HEK293 and HEK293/5I cells. However, the alaninyl-d4TMP formed in the HEK293/5I cells is almost entirely effluxed (Fig. 3C), whereas most of the alaninyl-d4TMP formed in the HEK293 cells accumulates intracellularly (Fig. 3D). The activated d4T metabolite, d4TMP, formed from alaninyl-d4TMP after cleavage of the phosphoramidate bond, was not detectable in the efflux media of either cell line and was found at low intracellular levels in the HEK293 cells.

Transport of PMEA by MRP4. We have shown that MRP5 extrudes PMEA in an unmodified form (Wijnholds et al., 2000), and we have verified that this is also the case for MRP4. After loading cells with 1 μM [^{3}H]bis-POM-PMEA, both efflux medium and intracellular fractions from MRP4-transduced and HEK293 parental cells were analyzed by HPLC (Table 2). PMEA was the only species detected in the efflux medium. After a 2-h incubation, HEK293/4.3 and HEK293/4.63 cells had effluxed 2.1- and 2.9-fold more PMEA

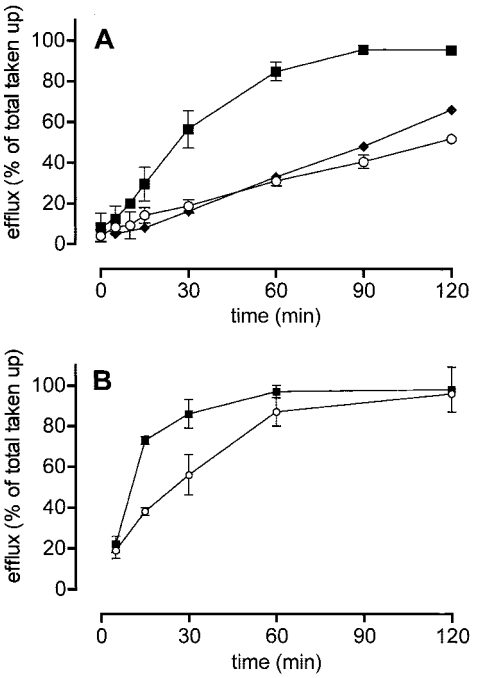


Fig. 2. Efflux from cells loaded with d4TMP prodrugs. Cells were loaded with 2.5 μM [^{3}H]So324 (A) or 0.27 μM [^{3}H]cycloSal-d4TMP (B) for 2 h under ATP-depletion conditions. Efflux from HEK293 (○), HEK293/5I (■), or HEK293/4.3 (♦) cells into complete medium was then measured and expressed as a percentage of the total drug accumulated during loading (150 pmol/10^6 cells for So324 and 0.5 pmol/10^6 cells for cycloSal-d4TMP). The values for So324 efflux are the mean of four determinations ± S.D.; those for cycloSal-d4TMP are the average of two determinations ± difference from the mean.

than the parental HEK293 cells, whereas the control clone HEK293/4.59, which does not express the transfected *MRP4* (Wielinga et al., 2002), showed efflux similar to that of the parental cells (Table 2). Intracellular concentrations of PMEA were decreased in the HEK293/4.3 and HEK293/4.63 cells, whereas intracellular concentrations of the phosphorylated derivatives were similar for all cell lines. We conclude that MRP4, like MRP5, extrudes PMEA in unmodified form.

Inhibition of MRP4- and MRP5-Mediated PMEA Efflux. Given the ability of MRP4 and MRP5 to cause resistance to some drugs, selective inhibitors that can inhibit the transporters in intact cells would be useful. We have tested a range of potential inhibitors on the initial rate of PMEA extrusion from cells preloaded with 1 μM [^{3}H]bis-POM-PMEA. Figure 4 shows that the initial rate of efflux from HEK293/4.3 (MRP4) and HEK293/5I (MRP5) cells was comparable, and considerably greater than that from the parental cells. This was not a consequence of different initial intracellular concentrations, as all three cell lines were loaded to the same extent (approximately 300 pmol/10^6 cells). The inset shows the relative rates of efflux mediated by MRP4 and MRP5 cells after subtraction of efflux from the parental line. The influence of various inhibitors (at subtoxic concentrations) on PMEA efflux is shown in Fig. 5, and the concentrations inhibiting MRP4 and MRP5 by 50% (IC_{50} values) are summarized in Table 3. Of the commonly used inhibitors of organic anion transport, probenecid inhibited PMEA efflux by MRP5 at much lower concentrations (IC_{50}, 200 μM) than those needed to inhibit MRP4 (IC_{50}, 2300 μM), as did sulfinpyrazone to a lesser extent (relative IC_{50} values 300 and 420 μM, respectively), whereas benzbromarone inhibited both transporters equally (Fig. 5A). In contrast, MK571 readily

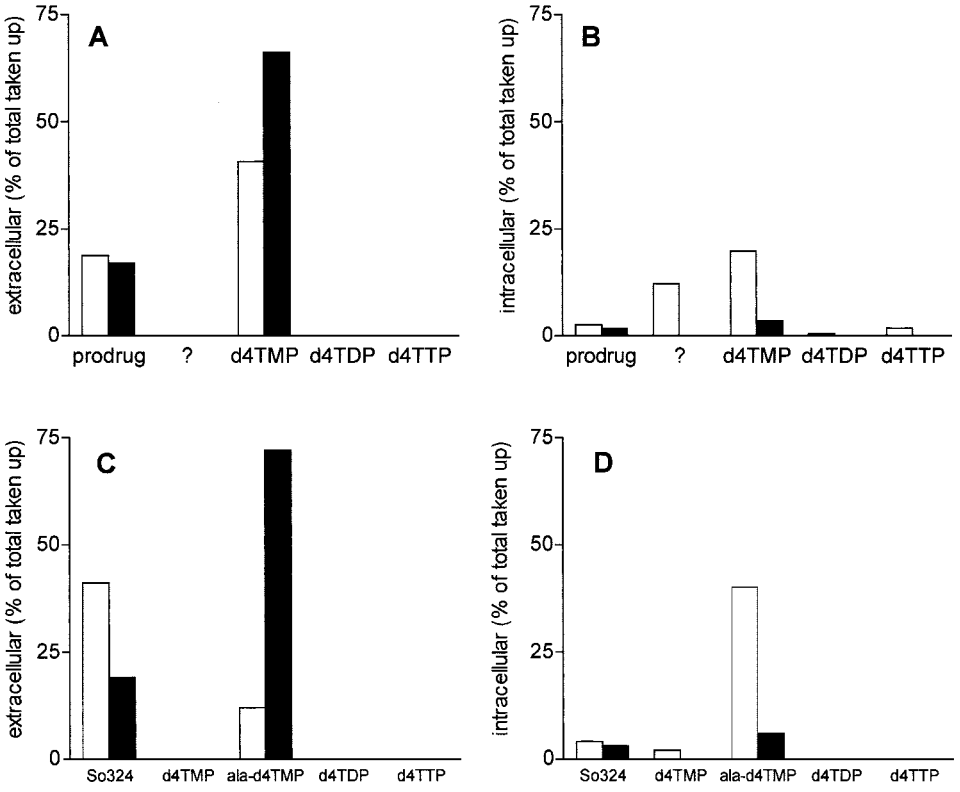


Fig. 3. HPLC analysis of cycloSal-d4TMP and So324 metabolites. Radiolabeled metabolites found in the efflux media (A, C) and cellular fractions (B, D) after 30 min of efflux from HEK293 (□) and HEK293/5I (■) cells loaded for 2 h with 0.27 μM [^{3}H]cycloSAL-d4TMP (A, B) or 2.5 μM [^{3}H]So324 (C, D). The total amount of radiolabel present in the cells after loading was the same as for Fig. 2, and efflux at 30 min is presented as a percentage of this value. Similar data were obtained in independent experiments of which here data of a representative experiment are shown.

TABLE 2

Analysis of the efflux of PMEA and metabolites from HEK293/MRP4 cells

Cells were loaded with 1 μM [3H]-bis(POM)PMEA and efflux was followed for 120 min. Intracellular and efflux samples were analyzed by HPLC. Values are expressed as a percentage of the total radioactivity loaded.

	PMEA		PMEAp		PMEApp		Total	
	0 min	120 min	0 min	120 min	0 min	120 min	0 min	120 min
				%				
Intracellular								
HEK293	99	48	N.D.	6	N.D.	24	100	78
4.59	99	51	1	4	N.D.	28	100	84
4.3	99	29	1	5	N.D.	25	100	59
4.63	99	19	1	4	N.D.	22	100	45
Extracellular								
HEK293	N.D.	22	N.D.	N.D.	N.D.	N.D.	N.D.	22
4.59	N.D.	16	N.D.	N.D.	N.D.	N.D.	N.D.	16
4.3	N.D.	41	N.D.	N.D.	N.D.	N.D.	N.D.	41
4.63	N.D.	55	N.D.	N.D.	N.D.	N.D.	N.D.	55

N.D., not detected.

inhibited MRP4 at 10 μM but was a poor inhibitor of MRP5. Based on the structural similarity between nucleotides and nucleosides, we also tested three inhibitors of nucleoside transport. Dipyridamole and dilazep inhibited MRP4-mediated PMEA efflux (IC_{50}, 2 and 20 μM, respectively), but MRP5 was inhibited by dipyridamole only at the highest concentration tested (Fig. 5B). NBMPR (at 100 μM) inhibited MRP4 but had no effect on MRP5 function (Fig. 5B).

We also tested inhibitors of phosphodiesterase 5 (Fig. 5C), because these were recently reported to be high-affinity inhibitors of MRP5 (Jedlitschky et al., 2000). Sildenafil and trequinsin both inhibited PMEA efflux mediated by MRP4 (IC_{50}, 20 and 10 μM, respectively) more effectively than that mediated by MRP5 (IC_{50}, 80 and 30 μM). In fact, only high concentrations of these compounds inhibited MRP5. Zaprinast, on the other hand, inhibited both pumps only at very high concentrations (IC_{50}, 250 μM for both MRP4 and MRP5). The P-glycoprotein inhibitor PSC833 and the breast cancer resistance protein-specific inhibitor Ko143 (Allen et al., 2002) had no effect on PMEA efflux mediated by either pump at concentrations of up to 1 μM (data not shown).

Vesicular Uptake by MRP4 and MRP5. Direct measurements of nucleoside analog drug transport by MRP4 and MRP5 into inside-out membrane vesicles have proven difficult. van Aubel et al. (2002) attempted unsuccessfully to show PMEA uptake into MRP4 containing vesicles. Similarly, using concentrations of up to 100 μM, we were unable to demonstrate transport of [^{3}H]PMEA, [^{3}H]PMEG, or [^{3}H]Fludara into vesicles derived from either HEK293/5I or HEK293/4.63 cells. Because cells overexpressing MRP5 transported alaninyl-d4TMP (Fig. 3C), we tested this substrate in vesicular transport. Using purified [^{3}H]alaninyl-d4TMP, we found ATP-dependent uptake with vesicles prepared from HEK293/5I, but not from parental HEK293 cells (Fig. 6A). At a concentration of 1 μM alaninyl-d4TMP, uptake by HEK293/5I vesicles reached a maximum of approximately 7 pmol/mg protein after 30 min. Although we did not have access to sufficient alaninyl-d4TMP to determine the K_m, transport did increase linearly with increasing substrate concentration (Fig. 6B) but was not saturated at the maximum concentration tested (100 μM). Consistent with the lack of efflux from MRP4 transfectants noted above, inside-out membrane vesicles derived from HEK293/4.63 cells did not accumulate [^{3}H]alaninyl-d4TMP.

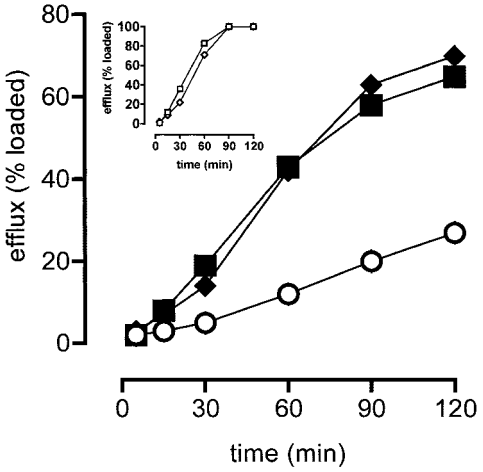


Fig. 4. PMEA efflux from bis-POM-PMEA-loaded HEK293 cells. After loading with 1 μM [^{3}H]bis-POM-PMEA under ATP-depletion conditions, efflux of PMEA equivalents from HEK293 (○), HEK293/4.3 (◆), and HEK293/5I cells (■) was measured over 2 h. Efflux is expressed as a percentage of the total [^{3}H]bis-POM-PMEA present in the cells immediately after the loading period, approximately 300 pmol/10^6 cells for all lines. The figure is representative of a frequently repeated assay. Inset, MRP4- and MRP5-dependent components of efflux are shown. These components represent efflux from HEK293/4.3 or HEK293/5I cells, respectively, minus efflux from parental HEK293 cells. The maximum (2 h) value was set at 100%, and all other values are a percentage of this. Each point and bar represent mean ± S.D. of three determinations.

The availability of a robust vesicular transport system for MRP5 made it possible to test the affinity of this transporter for analogs of nucleoside monophosphates in competition experiments. For comparison, we also tested MRP4 (Fig. 6C) using estradiol 17β-glucuronide ($E_2$17βG) as substrate, a compound not transported by MRP5 (unpublished observations). We tested this at the published K_m of 30 μM (Chen et al., 2001) to avoid the possibility that we would measure transport mediated by endogenous MRP1 in the HEK293 cells. As shown in Table 4, none of the nucleoside monophosphate analogs had a substantial effect on MRP4 or MRP5, even at a concentration of 1 mM. This confirms our results with intact cells showing that these compounds are, at best, low-affinity substrates of MRP4 and MRP5. Only with cPr-PMEDAP did we find 40% inhibition of MRP4. An unexpected finding, in light of a recent report (Jedlitschky et al., 2000), was that high concentrations of cGMP and cAMP were required to inhibit transport mediated by both transporters. The results in Table 4 also confirm the poor inhibition of MRP4 and MRP5 by 1 μM concentrations of the PDE5 inhibitors sildenafil and trequinsin, the maximal levels remaining soluble in the absence of serum proteins. Zaprinast inhibited both MRP4 and MRP5 equally and at concentrations lower than those required in intact cells, possibly because the pumps are more accessible to zaprinast in in vitro assays. Although the results of the growth inhibition assays suggest that the anticancer nucleoside analogs fludarabine, gemcitabine, and cytarabine are not substrates of MRP4 or MRP5, we tested this directly with the monophosphorylated forms. Slight inhibition of MRP4 (and no inhibition of MRP5) was observed at inhibitor concentrations of 1 mM, further evidence that these compounds are, at best, very low-affinity substrates of MRP4 and MRP5.

Discussion

Nucleoside analogs are important components of both anticancer and antiviral therapies. In general, these analogs must be taken up by target cells, activated by phosphorylation, and incorporated into nucleic acids to exert their toxic effects, processes mediated by a series of cellular and viral proteins. Changes in the expression of these proteins have been implicated in the development of resistance to nucleoside analogs (Galmarini et al., 2001). Although several resistance mechanisms have been identified in cell lines, these rarely correlate with clinical resistance (Galmarini et al., 2001). Recent reports demonstrating that the related ATP-binding cassette transporters MRP4 and MRP5 are able to transport nucleoside monophosphates raise the possibility that the extrusion of monophosphorylated nucleoside analogs from cells might contribute to resistance. To investigate this, we have characterized the transport of different classes of nucleoside analog drugs by MRP4 and MRP5.

In previous studies, the resistance to PMEA conferred by MRP4 overexpression was predicted to result from the efflux of PMEA itself, not phosphorylated metabolites (Schuetz et

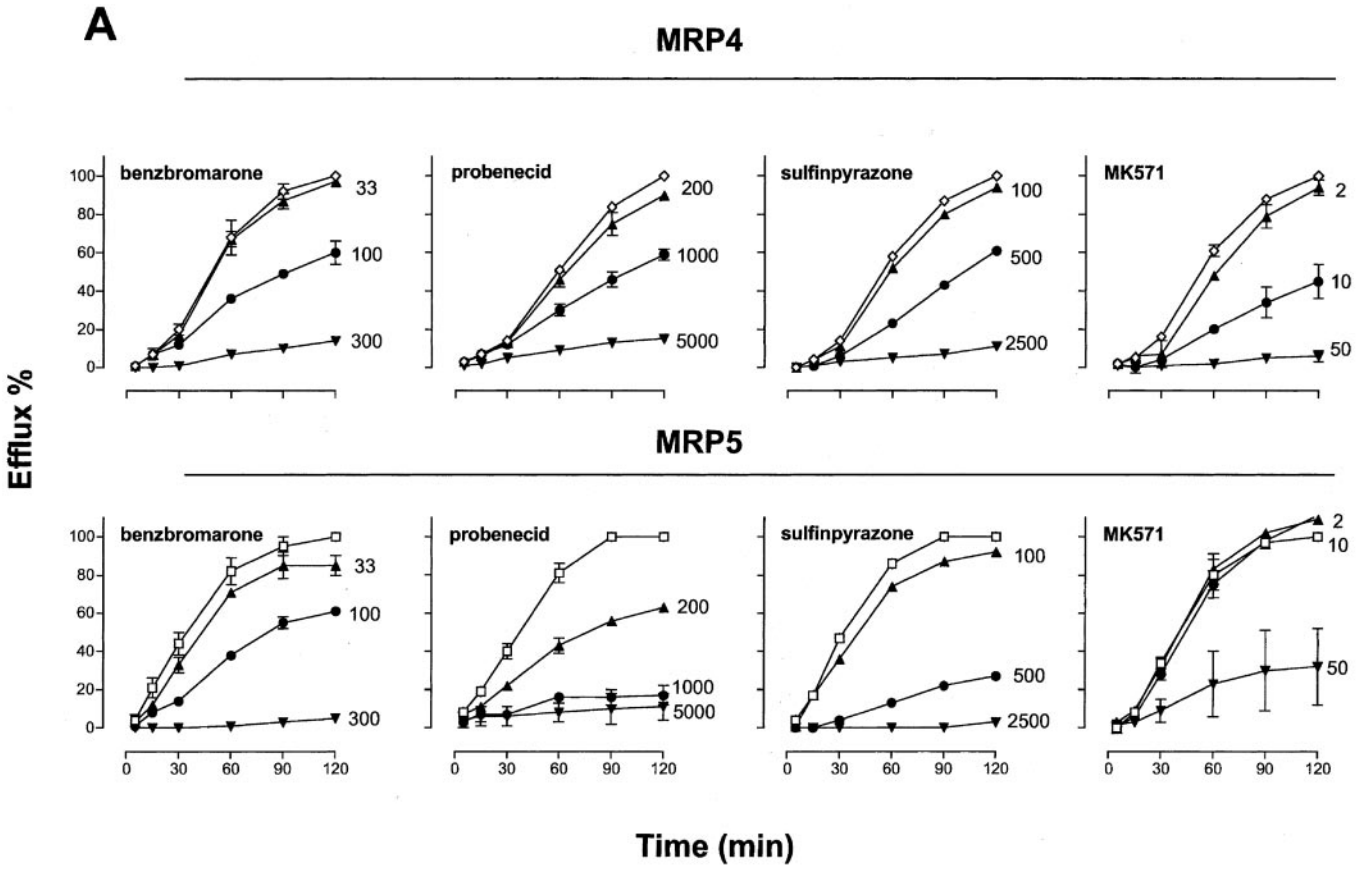
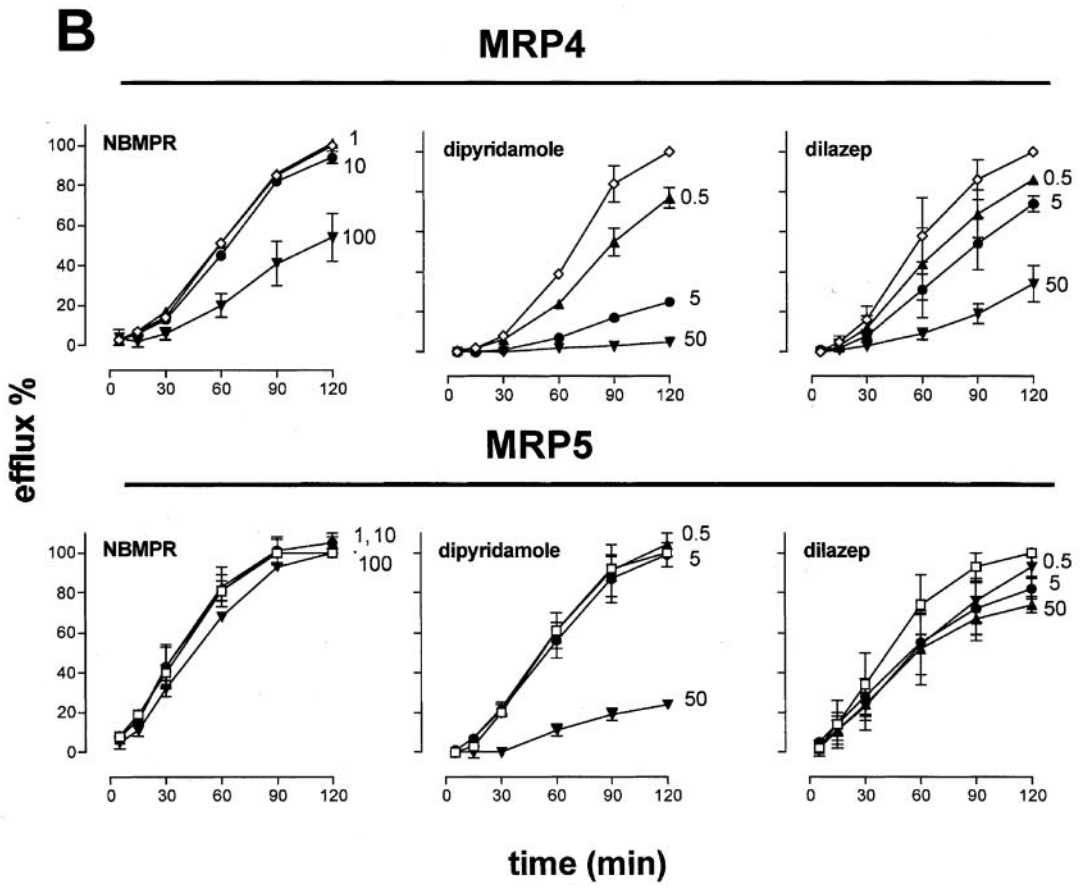


Fig. 5. Inhibition of MRP4- and MRP5-mediated PMEA efflux by drug transport inhibitors. The MRP4-dependent (◇, top) and MRP5-dependent (□, bottom) components of PMEA efflux from bis-POM-PMEA-loaded cells were determined as in Fig. 4, and represent the efflux from HEK293/4.3 or HEK293/5I cells, respectively, after subtraction of the efflux from parental HEK293 cells. Loading and efflux was carried out in the presence of the indicated (micromolar) concentrations of organic anion transport inhibitors (A), nucleoside transport inhibitors (B, facing page), or PDE5 inhibitors (C, facing page). Efflux at 2 h in the absence of inhibitor (open symbols) was set to 100%, and efflux in the presence of increasing concentrations of each inhibitor (▲, ●, ▼) is expressed as a percentage of these values. Each point and bar represent mean ± S.D. of three determinations.

al., 1999). We confirm this prediction here. Because MRP4 and MRP5 were previously shown to confer similar levels of resistance to PMEA (Lee et al., 2000; Wijnholds et al., 2000), we tested whether this was the case for other antiviral and anticancer nucleoside analogs. We found that MRP4 mediated high-level resistance against other acyclic nucleoside phosphonate drugs, whereas MRP5 did not. The 17-fold resistance to cPr-PMEDAP conferred by MRP4 on the HEK293 cells represents the most striking phenotype yet reported for MRP4-overexpressing cells. In contrast, MRP5, but not MRP4, mediated efflux from cells preloaded with the d4TMP prodrugs cycloSal-d4TMP and So324, with d4TMP and alaninyl-d4TMP representing the first substrates of MRP5 that are not measurably transported by MRP4. Transport of alaninyl-d4TMP, a dideoxyadenosine monophosphate prodrug, demonstrates that MRP5 recognizes dideoxynucleosides, although it should be stressed that HEK293 cells have additional unidentified endogenous transporters capable of effluxing d4TMP. The fact that alaninyl-d4TMP is transported shows that a free phosphate moiety is not a prerequisite for transport. Transport of alaninyl-d4TMP increased linearly as a function of concentration from 1 and 100 μM, suggesting a low-affinity interaction.

Like antiviral therapies, chemotherapy protocols for many types of cancer include nucleoside analogs, and resistance to these drugs represents a clinical problem. Previous studies investigating the ability of MRP4 or MRP5 to mediate resistance to anticancer nucleosides have yielded contrasting results. Chen et al. (2001) found no effect of MRP4 overexpression on the sensitivity of NIH3T3 cells to cladribine, whereas Davidson et al. (2002) found substantial resistance to cytarabine, gemcitabine, and cladribine in MRP5-overexpressing HEK293 cells. In contrast, we find low-level MRP4-mediated resistance against cladribine but no MRP4- or MRP5-mediated resistance against cytarabine, gemcitabine, or fludarabine. A possible explanation is that up- or down-regulation of nucleoside metabolizing enzymes in our HEK293 cells limits the potential for transport by MRP4 or MRP5. On the one hand, the toxicity of gemcitabine, cytarabine, and cladribine in cell lines increases proportionally with deoxycytidine kinase activity (Galmarini et al., 2001), and transfection with mitochondrial deoxyguanosine kinase has also been shown to increase sensitivity to the cytostatic activity of these agents (Zhu et al., 1998). On the other hand, transfection with human cytosolic nucleotidase imparts HEK293 cells with resistance against cladribine and gemcitabine (Hunsucker et al., 2001), and we have previously found that thiopurine nucleotides were rapidly dephosphorylated in HEK293 cells, limiting the toxicity of thiopurine drugs (Wielinga et al., 2002). All these examples show how strongly the levels of nucleoside monophosphate analogs in cells are influenced by the activity of enzymes producing or using these compounds.



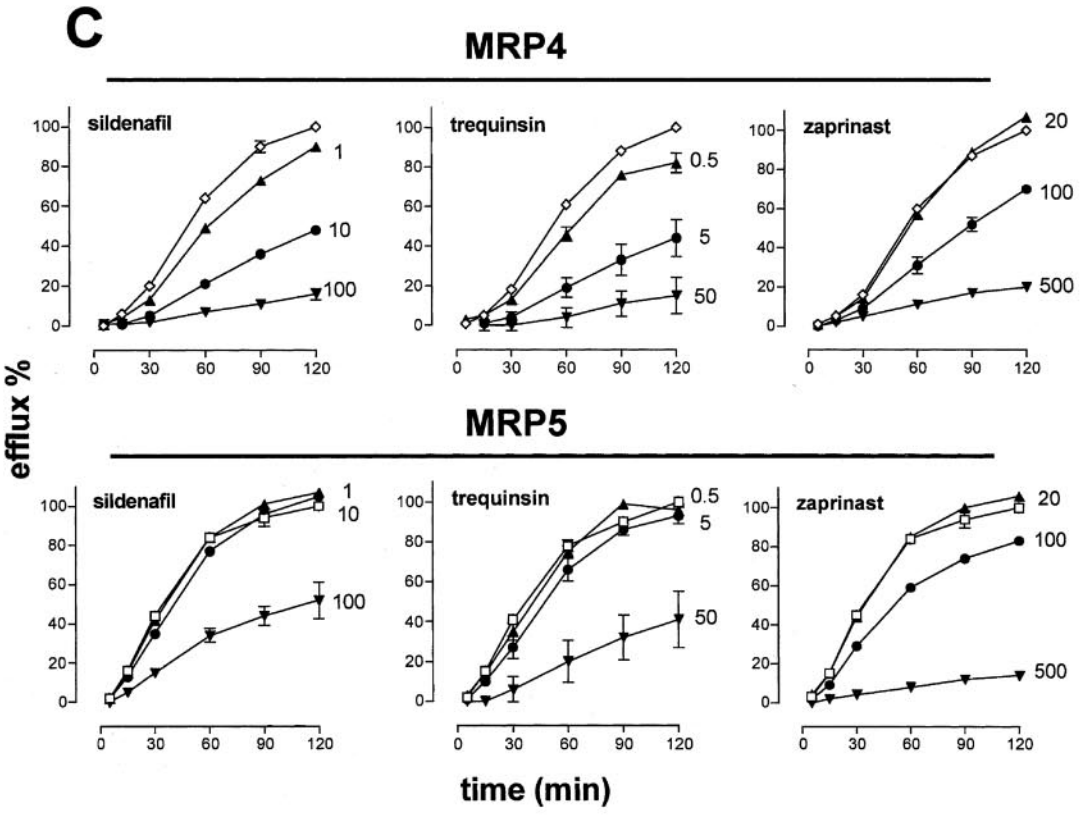


TABLE 3

Predicted IC_{50} values for inhibitors of MRP4- and MRP5-mediated PMEA transport

The rates of PMEA efflux over the first hour in Figure 2 were calculated and used to predict approximate IC_{50} values (micromolar) for MRP4- and MRP5-mediated PMEA efflux.

	IC_{50}	
	MRP4	MRP5
	μM	
Benzbromarone	150	150
Probenecid	2300	200
Sulfinpyrazone	420	300
MK571	10	40
Nitrobenzylmercaptopurine riboside	75	>100
Dipyridamole	2	30
Dilazep	20	>50
Sildenafil	20	80
Trequinsin	10	30
Zaprinast	250	250

It is therefore possible that MRP4 and MRP5 might be more important determinants of resistance in cells in which higher concentrations of transported substrates can accumulate than in the HEK293 cells used here.

Our results suggest that MRP4 or MRP5 is unlikely to make a major contribution to drug resistance in clinical practice, because both transporters have a relatively low affinity for their nucleoside monophosphate substrates. This was already indicated by studies with PMEA and thiopurines in intact cells, which suggested that intracellular substrate concentrations in the millimolar range are required to get substantial transport by MRP4 and MRP5 (Schuetz et al., 1999; Wijnholds et al., 2000; Wielinga et al., 2002). These results are confirmed by the vesicular transport experiments presented here. Although alaninyl-d4TMP is transported by MRP5 (Fig. 5), we have been unable to produce sufficient amounts of this compound to determine a K_m. None of the other nucleoside monophosphates available in radioactive form—PMEA, PMEG, or the monophosphate of fludarabine—was detectably taken up in vesicular transport experiments, even though PMEA is transported by MRP4 and MRP5 out of intact cells. The low affinity of these transporters for nucleoside monophosphate analogs is confirmed by our competition experiments: both PMEA and the monophosphates of gemcitabine, cytarabine, zalcitabine, and fludarabine inhibited MRP4- and MRP5-mediated transport by at most 20%, even at a concentration of 1 mM. Similarly, substantial inhibition of MRP4-mediated E_2-17βG transport was achieved only with 1 mM cPr-PMEDAP.

TABLE 4

Inhibition of MRP4- and MRP5-mediated substrate uptake into inside-out membrane vesicles by various compounds

[^{3}H]Estradiol 17β-glucuronide (30 μM) or [^{3}H]alaninyl-d4TMP (1 μM) was used to measure ATP-dependent transport mediated by MRP4 and MRP5, respectively. Uptake was carried out for 10 min for MRP4 and 15 min for MRP5. The MRP4- and MRP5-dependent components of uptake were determined by subtracting uptake in parental vesicles. Values are averages ± S.E. and are expressed as percentage of transport in the absence of inhibitor. The number of experiments is in parentheses.

Inhibitor	Concentration	Uptake by MRP4	Uptake by MRP5
	μM	*% of control*	
PMEA	1000	78 (1)	84 ± 10 (3)
cPr-PMEDAP	100	98 ± 5 (2)	N.D.
	1000	41 ± 10 (3)	99 ± 1 (2)
Cytarabine MP	1000	91 ± 10 (2)	126 ± 7 (2)
Fludarabine MP	1000	81 ± 4 (2)	105 ± 8 (2)
Gemcitabine MP	1000	96 ± 6 (2)	108 ± 7 (2)
ddCMP	1000	92 ± 8 (2)	99 ± 1 (2)
cGMP	100	87 ± 3 (2)	65 ± 2 (2)
	1000	48 ± 9 (3)	37 ± 5 (2)
cAMP	85	95 ± 10 (2)	104 ± 1 (2)
	850	73 ± 4 (3)	69 ± 9 (3)
Zaprinast	1	75 ± 15 (2)	73 (1)
	10	41 ± 11 (3)	57 ± 16 (3)
	100	7 ± 4 (2)	12 ± 6 (3)
Sildenafil	1	75 ± 1 (2)	101 ± 8 (2)
Trequinsin	1	73 ± 1 (2)	104 ± 9 (2)

N.D., not determined.

Recently, MRP4 and MRP5 were reported to transport cGMP with high affinity (Jedlitschky et al., 2000; Chen et al., 2001). In our vesicular transport experiments, however, uptake of cGMP was low and not consistently increased in MRP4- or MRP5-overproducing cells relative to untransfected control cells. In agreement with this result, we found that cGMP was a poor inhibitor of MRP4 and MRP5 activity in vesicular uptake experiments. Although cGMP slightly inhibited both pumps at 100 μM, a concentration of 1 mM was required to exceed 50% inhibition. Similarly, cAMP was a low-affinity inhibitor of both transporters. In the presence of 850 μM cAMP, MRP4 and MRP5 transport was inhibited by only 30 and 50%, respectively. The results of these vesicular transport experiments are in full agreement with our results with intact HEK293 cells, in which MRP4- and MRP5-mediated cyclic nucleotide efflux also exhibits characteristics of a low affinity transport process (P. R. Wielinga, I. van der Heijden, G. Reid, J. Beijnen, J. Wijnholds, and P. Borst, submitted).

It is unclear why we find a low affinity for cyclic nucleotides, whereas other labs report a high affinity. The two codon differences in the independently isolated MRP4 (Belinsky et al., 1998; Adachi et al., 2002; Lai and Tan, 2002) and MRP5 cDNAs (McAleer et al., 1999; Jedlitschky et al., 2000; Wijnholds et al., 2000) would not be expected to yield proteins with such dramatically altered substrate specificities and affinities. It is remarkable, however, that Chen et al. (2001, 2002) find a high-affinity uptake of radioactive cGMP in MRP4 vesicles, but very poor inhibition by cold cGMP of the uptake of other substrates such as E_2-17βG and metho-

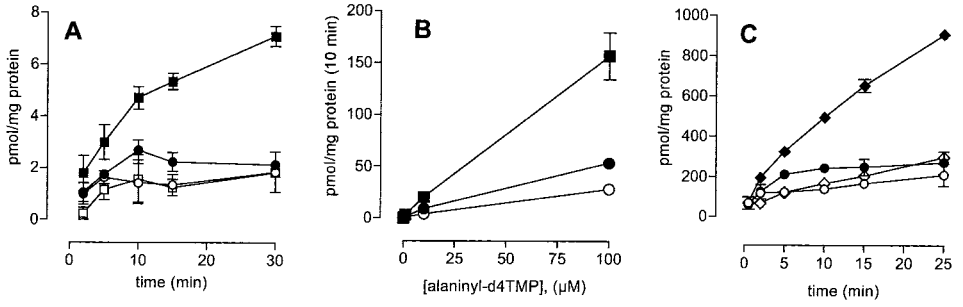


Fig. 6. Transport by membrane vesicles derived from HEK293 cells. Uptake of [^{3}H]alaninyl-d4TMP (A and B) by HEK293 (circles) or HEK293/5I (squares) vesicles, and uptake of [^{3}H]$E_2$17βG (C) by HEK293 (circles), and HEK293/4.63 (diamonds) vesicles, were measured in the presence of ATP (closed symbols) or AMP (open symbols). Time-dependence of alaninyl-d4TMP (A) and $E_2$17βG (C) uptake at 37°C are shown, with concentration-dependent alaninyl-d4TMP uptake shown in B. Data are given as the mean of three determinations ± S.D.

trexate. Chen et al. (2001, 2002) do not provide an explanation for this unusual discrepancy, but the results of their competition experiments are in line with our results. For MRP5, we can only speculate that the sodium butyrate treatment employed by Jedlitschky et al. (2000) to increase MRP5 expression in their transfectants coincidentally may have up-regulated the expression of an endogenous transporter(s) responsible for the high affinity cGMP transport observed in their vesicles, similar to the findings of Crane (2000). Because we were interested in the transport by MRP4 and MRP5 under more physiological conditions, we did not treat the cells with any gene expression modulating agents.

Another major discrepancy with published results is our finding that transport by MRP5 is rather insensitive to zaprinast, sildenafil, and trequinsin, inhibitors of cGMP phosphodiesterase 5 (PDE5). Whereas Jedlitschky et al. (2000) reported a K_i of 267 nM for the MRP5-mediated vesicular transport of cGMP, we see no effect of 1 μM sildenafil on PMEA transport. For the extrusion of PMEA from HEK293 cells, we find an approximate IC_{50} of 80 μM. The discrepancy is not a result of the substrate studied, because we saw no effect of 10 μM sildenafil on MRP-mediated efflux of cGMP from HEK293 cells (our unpublished results). If the inhibition of cGMP efflux by PDE5 inhibitors has physiological significance, as suggested by Jedlitschky et al. (2000), then it would probably be caused by inhibition of MRP4, which is more sensitive to these compounds (Table 2 and Fig. 5). MRP4 is also more sensitive than MRP5 to the nucleoside transporter inhibitors dipyridamole, dilazep and NBMPR (Table 2). Probenecid, and to a lesser extent sulfinpyrazone, are the only inhibitors found thus far that inhibit MRP5 more than MRP4.

Our demonstration that MRP4 and MRP5 transport nucleotide(s) (analogs) with low affinity raises the question of whether nucleotide transport is the main physiological function of these MRPs. For MRP4 this is unlikely, because we have recently found that this transporter has a high affinity for some steroid-sulfates and glucuronides (unpublished observations). It seems likely, therefore, that the transport of such compounds is a more important function of MRP4 than transport of nucleotides. For MRP5, a high-affinity substrate remains to be found.

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