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Review

The potential impact of drug transporters on nucleoside-analog-based antiviral chemotherapy

P. Borst [a,*], J. Balzarini [b], N. Ono [a], G. Reid [c], H. de Vries [a], P. Wielinga [a], J. Wijnholds [d], N. Zelcer [a]

[a] *Division of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands*
[b] *Rega Institute, Katholieke Universiteit Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium*
[c] *Children's Cancer Research Group, Department of Paediatrics, Christchurch School of Medicine, Christchurch, New Zealand*
[d] *Department of Ophthalmogenetics, The Netherlands Ophthalmic Research Institute, Meibergdreef 47, 1105 BA Amsterdam, The Netherlands*

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Abstract

Several ATP-binding cassette (ABC) transporters can transport drugs out of cells against steep concentration gradients resulting in resistance to the drugs transported. Recent work has shown that at least three members of the family of human Multidrug Resistance-associated Proteins (MRPs), MRP4, 5 and 8, are able to transport some nucleoside-monophosphate analogs. This can result in resistance to the base, nucleoside or nucleotide precursors of these results, at least in cell lines with high levels of transporter. The affinity of these transporters for the nucleotide analogs studied thus far is relatively low (millimolar rather than micromolar), and this limits their potential impact on the resistance. We briefly review how ABC transporters in general, and MRPs in particular, could affect the disposition and cellular accumulation of antiviral compounds.
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Keywords: ABC transporters; Cyclic nucleotides; MRPs; Nucleoside analogs; Oral drug availability

1. Introduction

The past 20 years has witnessed the characterization of a large number of membrane proteins that facilitate transport of drugs through membranes. Some of these drug transporters merely equalize the drug concentrations inside and outside our cells. Other transporters can transport drug against a drug concentration gradient by coupling drug transport to steep gradients of ions or metabolites. An example is the Na^+ gradient over the cell membrane used to drive inward transport. Another example is the organic anion transporter 1 (OAT1), which can mediate the uptake of cidofovir and adefovir into cells by coupling inward transport to the outward flux of dicarboxylates (Cihlar et al., 1999).

This review deals with drug transporters that can drive drug transport against a steep concentration gradient by coupling transport to hydrolysis of ATP. The known transporters of this type belong to the ATP-binding cassette or ABC transporter family (reviewed in Holland et al., 2003; Borst and Oude Elferink, 2002; Schinkel and Jonker, 2003). The human genome contains nearly 50 ABC transporters, but only a subset of these is known to be involved in drug transport. Although bacterial and plant ABC transporters are known to transport compounds into cells, all known mammalian drug transporters remove drugs from cells. The structure of the three main transporter types is presented in Fig. 1. The substrate specificity and tissue location of these drug transporters is summarized in Tables 1–4.

ABC transporters can influence antiviral drug therapy by their effect on drug pharmacokinetics and by promoting drug resistance. The effect of drug transporters on the distribu-

Abbreviations: Cf1093, aryloxyalaninylphosphoramidate of ddA-5′-monophosphate (ddAMP); cGMP, guanosine 3′,5′-cyclic monophosphate; cPr-PMEDAP, cyclopropyl-PMEDAP; *cyclo*SAL-d4TMP, cyclosaligenyl-d4T-5′-monophosphate; ddA, 2′, 3′-dideoxyadenosine; ddC, 2′, 3′-dideoxycytidine; d4T, 3′-deoxy-2′3′-didehydrothymidine; ddI, 2′,3′-dideoxyinosine; HEK, human embryonic kidney; HIV, human immunodeficiency virus; MRP, multidrug resistance protein; 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)

* Corresponding author. Tel.: +31-20-5122880; fax: +31-20-6691383.
E-mail address: P.Borst@nki.nl (P. Borst).

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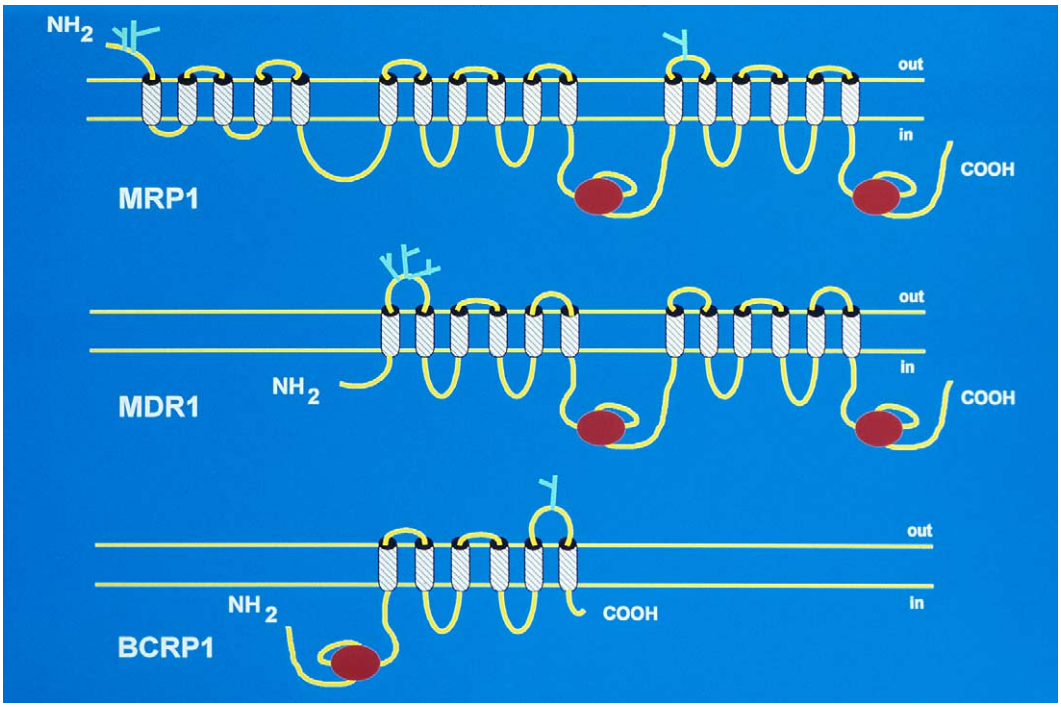

Fig. 1. Putative two-dimensional membrane topology of representative ABC transporters. See Borst and Oude Elferink (2002) for background references. MRP1, multidrug resistance (-associated) protein 1 (ABCC1); MDR1, the P-glycoprotein (ABCB1) encoded by the human MDR1 gene; BCRP1, the breast cancer resistance protein (ABCG2). BCRP1 functions as a homodimer.

tion of drugs in the body follows from the tissue distribution of these transporters (Table 2). P-glycoprotein (P-gp) (ABCB1), for instance, is located in the gut mucosa, where it hinders uptake of substrate drugs into the epithelial cells by directly transporting these drugs back into the gut content. Hence, gut P-gp has a major effect on the oral availability of substrate drugs, such as saquinavir and related human immunodeficiency virus (HIV) protease inhibitors (HPIs) (Huisman et al., 2000). The strategic position of P-gp in the blood–brain barrier, the blood–testis barrier, and the maternal–fetal barrier substantially affects the distribution of substrate drugs in the body (Borst and Oude Elferink, 2002; Schinkel and Jonker, 2003).

Table 1
ABC transporters handling (nucleotide analog) drugs

1. ABC (ATP-binding cassette) transporters are an ancient group of membrane proteins able to couple substrate transport to ATP hydrolysis resulting in vectorial transport

2. The main representatives in humans are:
   a. P-glycoprotein (P-gp, ABCB1, MDR1)
      Prefers neutral or basic amphipathic drugs
   b. The breast cancer resistance protein (BCRP1, ABCG2)
      Substrate preference, like P-glycoprotein, but more restricted
   c. The multidrug resistance (-associated) proteins (MRP1–9, ABCC1–6, 10–12)
      Prefer organic anions, such as drugs conjugated with GSH, glucuronide or sulfate, but MRP1 and 2 can transport some neutral drugs in the presence of GSH. MRP4, MRP5, and MRP8 can transport nucleotide analogs

A second ABC transporter present in the apical membrane of tissues important for drug handling (gut, liver, kidney, placenta) is ABCG2 (Borst and Oude Elferink, 2002; Schinkel and Jonker, 2003). This transporter is known under various other names, but is usually called breast cancer resistance protein (BCRP). ABCG2 can transport neutral compounds, such as HPIs. Recent work has shown, however, that ABCG2 can also transport organic anions, including methotrexate (MTX) (Volk et al., 2002; Chen et al., 2003) and sulfate conjugates of steroids (Suzuki et al., 2003). This suggests that ABCG2 could affect antiviral therapy more than is apparent at present.

P-gp and ABCG2 are both located in the apical membrane of polarized epithelia. This allows them to remove drugs from the body by transporting them into bile, urine, or gut contents. In contrast, a transporter, such as multidrug resistance (-associated) protein 1 (MRP1), is located basolaterally in polarized epithelia (Evers et al., 1996) and it transports drugs into the body. Nevertheless, MRP1 can prevent drug damage by protecting key precursor cells in bone marrow (Lorico et al., 1996, 1997; Wijnholds et al., 1997; Chen et al., 2001) and mucosa or skin (Wijnholds et al., 1998; Johnson et al., 2001). It is also used to protect body cavities, such as the content of the testicular tubules (Wijnholds et al., 1998) or the cerebrospinal fluid (Wijnholds et al., 2000a), where a basolateral transporter transports drug away from the cavity.

In this review, we concentrate on the multidrug resistance (-associated) proteins, or MRPs. The human MRP family

Table 2
Potential impact of drug transporters on antiviral drugs

1. Pharmacokinetics
   P-gp in gut mucosa, blood–brain barrier, placenta, ovary, testis
   BCRP1 in gut mucosa, placenta
   MRP1 in mucosa, blood–CSF barrier (choroid plexus), testis (Sertoli cells)
   MRP2 in gut mucosa, liver, blood–brain barrier

2. Providing sanctuaries
   P-gp in blood–brain barrier, placenta, ovary, testis
   BCRP1 in placenta?
   MRP1 in blood–CSF barrier (choroid plexus), testis (Sertoli cells), leukocytes
   MRP2 in blood–brain barrier?
   MRP4 in prostate?
   MRP4 and MRP5 in brain?

3. Selection for upregulation during long-term treatment?
(The virus profiting from host defense systems)

consists of nine members (Borst et al., 2000; Kruh et al., 2001; Jedlitschky and Keppler, 2002; Borst et al., 2003; Holland et al., 2003) and the ones characterized thus far are organic anion transporters. Tables 3 and 4 summarize the main locations and substrate specificities of these transporters. However, MRPs are more versatile than one would deduce from Tables 3 and 4. MRP1 and 2, for instance, cannot only transport organic anions, such as compounds conjugated to glutathione, glucuronate, or sulfate, but they can also transport neutral compounds in the presence of glutathione, metal ions, like cisplatin, arsenite and antimonite (possibly in complex with glutathione) and glutathione itself.

2. Transport of antiviral nucleoside/nucleotide analogs by MRP4 and MRP5

The first indication that MRP5 might be able to transport nucleotide analogs came from Wijnholds et al. (1999), who found that cells transfected with an *MRP5* gene construct were resistant to thiopurines. Schuetz et al. (1999) showed that cells selected for resistance to adefovir (PMEA) overproduced MRP4 and subsequent work has established that MRP4 (Schuetz et al., 1999; Chen et al., 2001; Kruh et al., 2001; Adachi et al., 2002a,b; Lai and Tan, 2002; Wielinga et al., 2002; Reid et al., 2003a) and MRP5 (Wijnholds et al., 2000b; Jedlitschky et al., 2000; Reid et al., 2003a) can confer resistance to a range of base, nucleoside, and nucleotide analogs, as summarized in Table 5. For the thiopurines and PMEA a detailed metabolite analysis has shown that resistance is due to transport by MRP4/5 of the nucleoside-monophosphate, but not the base, nucleoside, nucleoside-diphosphate or -triphosphate (Wielinga et al., 2002; Reid et al., 2003a). By inference, we assume that the same holds for all other compounds listed in Table 5.

The resistance levels in Table 5 are low and there are at least three reasons for this:

Table 3
The MRP family

	Size[a]	Tissue distribution	Apical or basolateral	Organic anion pump
MRP1	Long	Wide	B	+
MRP2	Long	Liver, gut, kidney	A	+
MRP3	Long	Liver, gut, adrenal, etc.	B	+
MRP4	Short	Wide	A/B	+
MRP5	Short	Wide	B	+
MRP6	Long	Liver, kidney, other tissues?	B	+
MRP7	Long	Wide	?	+
MRP8	Short	Wide	?	+
MRP9	Short	Wide	?	?

[a] MRPs come in two forms, the long one depicted in Fig. 1, and a short one lacking the N-terminal domain, also absent in the MDR1 P-glycoprotein.

Table 4
MRPs: substrates, resistance, KO phenotype

	Preferred substrates	Transport of				KO phenotype
		MDR drugs	MTX	NMP analogs	Cisplatin	
MRP1	GS-X, Gluc-X	+++	+	−	−	Reduced flammatory response (LTC4)
MRP2	GS-X, Gluc-X	+	+	−	+	Dubin–Johnson syndrome[a]
MRP3	Gluc-X, Sulf-X	+	+	−	−	None thus far
MRP4	cGMP, cAMP, Gluc-X, Sulf-X, PGEs	−	+	+	−	None thus far
MRP5	cGMP, cAMP, GS-X	−	−	+	−	None thus far
MRP6	GS-X, BQ123	+[b]	−	−	+[b]	Pseudoxanthoma elasticum[a]
MRP7	Gluc-X, GS-X?	−	−	−	−	?
MRP8	cGMP, cAMP	−	?	+	?	?

[a] Human disease caused by total absence of transporter.
[b] Low level.

Table 5
Resistance against drugs caused by MRP4 and MRP5 in transfected cells (taken from Reid et al., 2003a)

Base/nucleoside/nucleotide	HEK293	Resistance factor	
	IC_{50} (μM)	MRP4	MRP5
Altered purine ring			
6-Mercaptopurine	3	3	3
Thioguanine	1	3	2
Altered pyrimidine ring	No resistance found		
Purine nucleoside analog			
Adefovir I (PMEA)	79	3	3
PMEG	2	4	1
PME diaminopurine (PMEDAP)	68	5	2
cPr-PMEDAP	2	10	1
Didanosine (ddI)	>250	n.d.	n.d.
ddA	>400	1	1
Abacavir (ABC)	250	2	2
Cladribine	2	2	1.5
Ganciclovir[a] (GCV)	12	5	n.d.
Pyrimidine nucleoside analog			
Zidovudine[b] (AZT)	1800	n.d.	1
Stavudine[b] (d4T)	1000	1	1
Zalcitabine (ddC)	>1000	1	n.d.

[a] From Adachi et al. (2002a), using CEM-r1-TK cells.
[b] Some resistance found by Schuetz et al. (1999) in a PMEA-selected MRP4-overproducing T-lymphoid cell line.

1. First and foremost is the low affinity of MRP4/5 for all nucleotide analogs tested thus far. This has been demonstrated in vesicular transport experiments (Reid et al., 2003a), but it also follows from the relatively high intracellular nucleotide analog concentrations required for a difference between resistant and sensitive cells. In fact, the human T-lymphoid cell line CEM-r1, selected for high resistance to PMEA, has a partial defect in mitochondrial adenylate kinase, in addition to high overexpression of MRP4 (Schuetz et al., 1999). The adenylate kinase is required for phosphorylation of PMEA. Hence, PMEA accumulates to high levels in these cells.
2. The HEK293 cells often used to screen for MRP function have a high intrinsic resistance to several nucleoside analogs. This is not due to absence of nucleoside transporters (unpublished results), but presumably to sluggish phosphorylation of the nucleoside analogs in HEK293 cells. This may explain why the HEK cells are not affected by zidovudine, stavudine, and 3TC (Reid et al., 2003a), whereas the MRP4-overproducing CEM-r1 cells of Schuetz et al. (1999) are partially resistant to the anti-HIV activity of these drugs. Even in the CEM-r1 cells, however, substantial resistance to the cytostatic effect of ganciclovir was only obtained when the CEM-r1 and the control parental cells were both transfected with a herpesvirus thymidine kinase gene construct to allow efficient phosphorylation of ganciclovir (Adachi et al., 2002b).

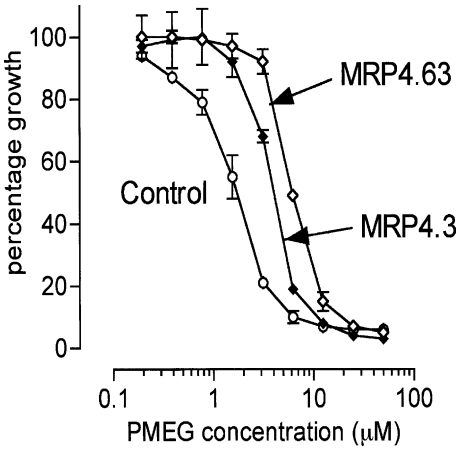

Fig. 2. Resistance of HEK293 (control) cells and two different HEK293 cell lines transfected with an *MRP4* gene construct to PMEG [9-(2-phosphonomethoxyethyl)guanine]. The MRP4 level in MRP4.63 is about twice that in MRP4.3. Cytotoxicity was tested in a standard growth assay, as described in Reid et al. (2003a).

3. The degree of resistance is strictly dependent on the degree of overexpression of the transporter studied in the transfected cell. This is obvious from Fig. 2, in which the HEK4.63 cells give a higher level of resistance to PMEG than the HEK4.3 cells which contain about twofold less MRP4 (Wielinga et al., 2002). An additional complication is that not all the transporter molecules made in the transfected cell reach the plasma membrane where they can contribute to resistance. The fraction trapped in the cell can be high and is difficult to quantitate (Borst et al., 1999). This may explain why groups at Eli Lilly (Davidson et al., 2002; Dantzig et al., 2003) have observed higher levels of resistance in the *MRP5*-transfected HEK293 cells produced in their laboratory than we have seen in our laboratory. For 6-thioguanine they find a resistance factor of 9, where we get 2 (Table 5). They also find resistance for gemcitabine, cytarabine, cladribine, and 5-fluorouracil (5-FU). The 5-FU resistance appears due to transport of 5-F-2′-dUMP. In vesicular transport experiments the authors find a K_m of 1.3 mM for this substrate, confirming that MRP5 is a poor nucleotide analog transporter. The details of these results remain to be published.

HEK293 cells are highly resistant to the cytostatic effect of d4T and they are not inhibited by the recently synthesized arylphosphoramidate precursors of d4TMP either. We have shown, however, that both d4TMP and especially alaninyl-d4TMP, which is released from the prodrug prior to d4TMP formation (Balzarini et al., 1996), are transported by MRP5, albeit with low affinity (Reid et al., 2003a).

3. Transport of cyclic nucleotides by MRP4 and MRP5

The low affinity of MRP4 and MRP5 for nucleotide analogs raises the question whether these transporters are

only low-affinity high-capacity junk removers, somewhat like the glutathione-*S*-transferases. Phrased differently, are there physiological substrates transported with high affinity by MRP4 and MRP5? The report by Jedlitschky et al. (2000) that MRP5 transports cGMP with micromolar affinity (and cAMP with much lower affinity) seemed to settle the issue: MRP5 is a cyclic nucleotide pump and the transport of nucleotide analogs is only a side activity. This conclusion was reinforced by analogous results reported for MRP4 (Chen et al., 2001). Unfortunately, this conclusion is not unchallenged, as our group only finds low-affinity transport of cyclic nucleotides by MRP4 and MRP5 (Reid et al., 2003a; Wielinga et al., 2003). We have raised the possibility that the results in other laboratories are attributable to inadvertent upregulation of an endogenous transporter (not endogenous MRP4 or MRP5) in the transfected cells used by our colleagues. As long as this putative endogenous transporter (MRP8 or MRP9?) has not been identified, the matter remains unresolved.

However, we have found a range of other physiological substrates for MRP4 that are transported with micromolar affinity. The list now includes steroid-glucuronides and steroid-sulfates (Zelcer et al., 2003b), hydroxylated bile salts conjugated to glucuronide (unpublished results), and prostaglandins E_1 and E_2 (Reid et al., 2003b). There is clearly no lack of high-affinity substrates for MRP4. Whether this transporter is normally not too occupied with physiological substrates to transport nucleotide analogs or cyclic nucleotides remains to be seen. For MRP5 no high-affinity substrate is known yet.

4. Transport of antiviral nucleotide analogs by MRP8 and MRP9

The two latest additions to the MRP family, MRP8 (Tammur et al., 2001; Yabuuchi et al., 2001; Bera et al., 2001) and MRP9 (Tammur et al., 2001; Yabuuchi et al., 2001; Shimizu et al., 2003; Bera et al., 2002) resemble MRP4 and 5 most (Tammur et al., 2001). They might therefore also be able to transport nucleotide analogs. It has been difficult to get high-level expression of MRP8 in the standard cell lines used in our laboratory, but recently Guo et al. (2003) managed to get expression of MRP8 in a pig LLC-PK1 kidney cell line. As shown in Table 6, these cells are resistant to PMEA as well as ddC, 5-FU, and 5-fluoro-2′-deoxyuridine (5-FUdR), but not to 6-TG, CdA, DCF, 3TC, or AZT. Membrane vesicles from MRP8-overproducing cells transport 5-FU-dUMP, suggesting that MRP8 causes resistance by transporting nucleotide analogs out of the cell, like MRP4 and 5. Whether MRP8 also has a low affinity for these substrates remains to be determined. The substrate specificity of MRP9 has not yet been determined.

Table 6
Drug sensitivity of MRP8-transfected pig kidney cells (LLC-PK1) (modified from Guo et al., 2003)

Drug	IC_{50} of parental cells (μM)	Fold resistance in MRP8 transfectant
PMEA	0.5	5.4
6-TG	0.5	1.1
ddC	0.4	6.1
5-FU	0.01	2.9
5-FUdR	0.02	5.2

No resistance was found for CdA, DCF, 3TC, or AZT.

5. Conclusions and outlook

Farmacotherapy Anno 2003 is still empirical, even though we spend increasing amounts of time teaching students about rational drug design. As we learn more about the vagaries of drug uptake and disposition, we start to realize the hurdles that our precious drugs have to take on their road to cellular targets. Daunting obstacles are the ABC transporters lurking in the cell membranes ready to pump out toxic molecules from cells, or intercept them in the lipid bilayer as they try to get in.

As summarized in Table 2, ABC transporters may potentially interfere with chemotherapy at three levels. Interference of P-gp with the uptake of HPIs from the gut is well documented (Borst and Oude Elferink, 2002; Schinkel and Jonker, 2003); the role of ABCG2 and of MRP2 is still uncertain. MRP2 is an interesting potential player. This organic anion transporter is best known for its essential role in the excretion of bilirubin-glucuronides from the liver into the bile (Oude Elferink et al., 1997), but MRP2 is also present in the apical membrane of intestinal epithelium, where it can inhibit uptake of some carcinogens by transporting them back into the gut lumen (Dietrich et al., 2001a,b,c). MRP2 also contributes to the blood–brain barrier (Potschka et al., 2003). Huisman et al. (2002) have recently shown that MRP2 can transport the HPI saquinavir and that transport can be stimulated by unrelated compounds, such as probenecid. MRP2 has a remarkable ability to undergo allosteric stimulation by a variety of compounds. Some of these are physiological, such as steroid-glucuronides (Bakos et al., 2000; Bódó et al., 2003; Zelcer et al., 2003a). This makes it difficult to predict what the contribution of MRP2 could be to the disposition of HPIs.

Besides HPIs, one may expect that other antivirals that are neutral or basic will be affected by P-gp and/or ABCG2. In fact, there is indirect evidence that bis(POM)PMEA is a P-gp substrate (Balzarini et al., 1991). With increasing attempts to make cell-permeable antiviral nucleotide precursors, the problems created by P-gp preventing these compounds from getting into the body, into sanctuaries, or into cells, will probably increase.

The precise role of MRP4, MRP5, MRP8, and possibly MRP9 in limiting cellular accumulation of antiviral nucleotide analogs, remains to be established. As pointed out before, the low affinity of MRP4 and 5 for the nucleotide

analogs studied thus far, makes a prominent role of these transporters in antiviral resistance less likely at present. It should also be emphasized that viruses multiply in normal cells, which are highly resistant to the DNA rearrangements leading to overexpression of ABC transporter genes in cancer cells. However, viruses might activate stress responses resulting in increased expression of transporters, and some cell types may normally contain high levels of certain MRPs, providing sanctuaries for virus replication. These are speculations that remain to be tested. As it stands, there is no evidence that antiviral chemotherapy in patients is adversely affected by any MRP.

The KO mice lacking one or more of these transporters could, in the long run, provide more solid information on the ability of MRPs to contribute to resistance to antiviral nucleosides/nucleotides. We have not observed altered pharmacokinetics of PMEA in the Mrp5 (−/−) mice (unpublished results), but it is possible that this negative result is caused by compensatory activity of related transporters. In collaboration with Dr. John Schuetz (Memphis, USA) we have generated an *Mrp5*/*Mrp4* double KO mouse to investigate this issue.

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