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INVITED REVIEW

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Multidrug resistance-associated proteins 3, 4, and 5

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Abstract We summarize in this paper the recently published results on multidrug resistance-associated proteins 3, 4, and 5 (MRPs 3–5). MRP3 can transport organic compounds conjugated to glutathione, sulfate, or glucuronate, such as estradiol–17β-glucuronide, bilirubin–glucuronides, and etoposide–glucuronide, and also bile salts and methotrexate. Studies in knockout mice have shown that Mrp3 contributes to the transport of morphine–3-glucuronide and acetaminophen–glucuronide from the liver into blood. There is no evidence for a major role of MRP3 in bile salt metabolism, at least in mice. The function of MRP3 in other tissues, notably the gut and the adrenal cortex, remains to be defined. MRP4 and MRP5 have attracted attention by their ability to transport cyclic nucleotides and many nucleotide analogs. The initial reports that MRP4 and 5 can transport cGMP with μM affinity have not been confirmed in recent work and the physiological importance of cyclic nucleotide transport by MRP4 and 5 remains to be determined. Transfected cells containing high concentrations of MRP4 and 5 are moderately resistant to base, nucleoside, and nucleotide analogs. The affinity of both transporters for nucleotide analogs is low (K_m around 1 mM) and there is no evidence that the transport of these compounds results in resistance in vivo. The physiological function of MRP4 and 5 remains to be found.

Abbreviations 6MP: 6-Mercaptopurine · 6TG: 6-Thioguanine · 5-FudR: 5-Fluorodeoxyuridine · ABC: ATP-binding cassette · BCRP: Breast cancer resistance protein · BDL: Bile duct ligation · BSEP: Bile salt export pump · CAR: Constitutive androstene receptor · CDCF: Dicarboxy-dichlorofluorescein diacetate · CFTR: Cystic fibrosis transmembrane conductance regulator · CMFDA: 5-Chloromethylfluorescein diacetate · DHEAS: Dehydroepiandrosterone-3-sulphate · DNP–GS: Dinitrophenyl-S-glutathione · $E_2$17βG: Estradiol–17β–glucuronide · FXR: Farnesoid-X-receptor · GSH: Glutathione · HC: Hyocholate · HDC: Hyodeoxycholate · HEK293: Human embryonic kidney cells 293 · K_i: Inhibitory constant · K_m: Michaelis constant · KO: Knockout · MDCK cells: Madin Darby canine kidney cells · M3G: Morphine–3-glucuronide · MK571: 3-([3-(2-[7-Chloro-2-quinolinyl]ethenyl)phenyl-(3-dimethylamino-3-oxopropyl)-thio-methyl]thio)propanoic acid · MRP: Multidrug resistance-associated protein · MTX: methotrexate · NPPB: 5-nitro-2-(3-Phenylpropylamino) benzoic acid · PGE: Prostaglandin E · PMEA: 9-(2-Phosphonylmethoxyethyl)adenine · PXR: Pregnane-X-receptor · SNP: Single nucleotide polymorphism · V_{max}: Maximal velocity · WT: Wild type

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Introduction

One of the largest sub-families of the ATP-binding cassette (ABC) transporters able to affect drug disposition is the ABCC (multidrug resistance-associated protein, MRP) family. There are now nine members of the multidrug resistance-associated protein family and eight of these (MRP1–8) are known to be organic anion transporters. Between them, the MRPs can transport a large range of organic anions, including anionic drugs and drugs conjugated to glutathione (GSH), sulfate, or glucuronate. No two MRPs have exactly the same substrate specificity or tissue distribution and the precise function of most of the MRPs remains to be established. The MRPs have been reviewed by us [13–19] and others [1, 11, 20, 35, 41, 46, 50, 54, 55, 71, 78] in recent years and a comprehensive review by Deeley et al. is in press in *Physiological Reviews* (2006). In this paper, we discuss recent data on MRP3, 4,

and 5. The other MRPs are discussed in other reviews in this volume.

MRP3 (ABCC3)

MRP3 consists of 1,527 amino acids [8] and its predicted membrane topology resembles that of MRP1. Among the MRP family members, MRP3 shares the highest degree of amino acid homology with MRP1 (58%) [8, 47, 53]. MRP3 was, therefore, initially expected to transport a similarly broad spectrum of anti-cancer agents as MRP1 and to contribute to clinically relevant drug resistance. The early finding that rat Mrp3 is able to transport some bile salts also led to speculations that MRP3 would be involved in bile salt metabolism. Neither of these conjectures was borne out of experimental data, however. It now appears that MRP3 is involved in transporting some endogenous glucuronosyl conjugates, such as bilirubin–glucuronides, and conjugated drugs out of cells, but other functions may still turn up.

Substrate specificity of MRP3

MRP3 is a typical organic anion transporter able to transport organic compounds conjugated to GSH, sulfate, or glucuronate. The vesicular transport system has been extensively used to characterize its substrate specificity (Table 1). MRP3 has a preference for glucuronidated compounds with morphine–3-glucuronide (M3G) [111], etoposide–glucuronide [110], estradiol–17β-glucuronide ($E_2$17βG) [37, 110, 114], and bilirubin–glucuronides [9, 60, 112] being examples of excellent substrates. MRP3/Mrp3 transports several bile salts although the actual rate of transport is species dependent with rat Mrp3 transporting several bile salts with high affinity and at a high rate [38], whereas human MRP3 is less proficient in this respect [109]. However, the catalytic efficiencies (V_{max}/K_m) for the transport of the glucuronidated bile salts hyocholate–glucuronide and hyodeoxycholate–glucuronide by human MRP3 are the highest reported for any MRP3 substrate to date [112]. The physiological significance of this high efficiency remains to be tested.

Unlike MRP1 and MRP2, MRP3 appears unable to transport GSH [53]. Cells with high MRP3 levels contain normal GSH concentrations and transport of neutral natural product agents by MRP3 does not seem to require GSH, as buthionine sulfoximine does not attenuate MRP3-mediated resistance to etoposide [110]. The affinity of MRP3 for methotrexate (MTX) is low [37, 110, 114], consistent with the fact that MRP3 only confers resistance during short-term exposure to high MTX concentrations [53].

Transport by MRP1 and MRP2 can be stimulated by several compounds [10, 26, 107] and this also holds for MRP3. Compounds shown to increase MRP3-mediated transport of $E_2$17βG are the sulfate conjugates ethinyl estradiol–sulfate (fourfold) [26], E3040 sulfate (1.5-fold) [37], and 4-methylumbelliferone–sulfate (fivefold) [37]. Whether stimulated transport has any physiological relevance [18] remains to be seen, however.

Tissue distribution and induction of MRP3

Compared to MRP1, MRP3 has a restricted tissue distribution pattern. *MRP3* is expressed in liver, pancreas, small intestine, and colon with lower amounts of mRNA detected in bladder, kidney, pancreas, lung, spleen, stomach, and tonsils [8, 47, 51, 53]. The presence of MRP3 protein has been confirmed in liver, kidney, the intestine, adrenals, pancreas, gallbladder, pancreas, and spleen [86]. In polarized epithelial cells, MRP3 localizes to the basolateral membrane [86]. There is controversy about the actual amount of MRP3 that is present in human liver. Several studies detected high levels of *MRP3* mRNA in human liver [8, 47, 51, 52]. Immunohistochemistry of normal human liver, however, showed only prominent MRP3 staining in cholangiocytes with some weak staining of the hepatocytes around the portal tract [86]. These contrasting results might be explained by a recent study of Lang et al. [57] who found that hepatic MRP3 expression varies (up to 80-fold) among individuals. Part of this variation could be linked to a single nucleotide polymorphism in the promotor region of *MRP3* leading to diminished binding of nuclear factors. In normal rat liver, Hirohashi et al. [36] only detected low Mrp3 mRNA levels, whereas in mouse liver, Zelcer et al. [112] found high Mrp3 levels by immunoblot and immunohistochemistry analysis. This difference between rat and mouse may be partly strain dependent, as we recently found that hepatic Mrp3 levels vary among different mouse strains (unpublished results).

MRP3 in the liver is highly inducible. In humans [86, 116] and rats [30, 36, 70], MRP3/Mrp3 is up-regulated during cholestasis and in the absence of functional MRP2/Mrp2, as seen in patients with the Dubin–Johnson syndrome [51] and Eisai hyperbilirubinemic rats [36]. This shows that MRP3 is induced in these species when the canalicular route for the excretion of organic anions is blocked. In mice, the inducibility of Mrp3 is less pronounced, with no or modest up-regulation during bile duct ligation (BDL), depending on the mouse strain [12, 112], or in the absence of Mrp2 (Marijn Vlaming and Alfred Schinkel, personal communication). As basal levels of Mrp3 are high in mice compared to rats, there may be little room for up-regulation.

The induction of MRP3 in the liver is thought to proceed via receptor-mediated pathways including several nuclear receptors [33, 39, 62, 105]. An important nuclear receptor is the pregnane-X-receptor (PXR). Hepatic induction of Mrp3 by PXR activators is absent in *Pxr* knockout (KO) mice [95], indicating that this nuclear receptor regulates *Mrp3* expression. The involvement of the constitutive androstene receptor (CAR) in MRP3 induction is doubtful as Cherrington et al. [25] still found hepatic Mrp3 induction in *Car* KO mice treated with the prototype CAR activator phenobarbital. The direct involvement of

Table 1 In vitro substrates for MRP3, MRP4, and MRP5

Substrates	K_m (μM)	V_{max} (pmoles min^{-} mg^{-} protein)	Reference
MRP3			
Glucuronides			
$E_2$17βG	67, 18, 26	415, 474, 76	[37], [110], [114]
Ethinylestradiol–glucuronide	9	58	[26]
Etoposide–glucuronide	11	138	[110]
E3040–glucuronide[a]	+	+	[37]
Morphine–3-glucuronide		500–1000	[111]
Acetaminophen–glucuronide	900	2100	[105]
Bilirubin–glucuronide[a]	+	+	[60]
Glutathione conjugates			
LTC_4	5	20	[114]
GS–DNP	6	4	[114]
Bile salts (and conjugates)			
Taurocholate	16	50	[38]
Glycocholate[a]	+	+	[38]
TLC–sulfate	3	162	[38]
Hyocholate–glucuronide	0.2	200	[112]
Hyodeoxycholate–glucuronide	0.7	400	[112]
Miscellaneous			
MTX	776	228	[114]
MRP4			
Conjugated steroids			
$E_2$17βG	30	102	[24]
DHEAS	2	45	[108]
Prostanoids			
PGE_1	2.1	6.9	[77], [81]
PGE_2	3.4, 3.5	6.4, 3.3	[77], [81]
TXB_2	9.9	51	[81]
$PGF_{2\alpha}$	12.6	46	[81]
Cyclic nucleotides			
cAMP	45	4	[24]
cGMP	10, 180, 170	2, 58	[24], [85], [98]
Antimetabolites			
MTX	1300, 220	430, 240	[23], [98]
Leucovorin	640	1950	[23]
Bile salts[b]			
Cholate	14.8	75	[79]
Taurocholate	3.8, 7.7	154	[79], [80]
Glycocholate	25.8	175	[79]
Miscellaneous			
Folate	170	680	[23]
Urate	1550	47	[98]
p-Aminohippurate	160	80	[91]
MRP5			
Cyclic nucleotides			
cAMP	379		[42]
cGMP	2.1		[42]
Antimetabolites			
5-FdUMP (5-FUMP, dUMP)[a]	1100	439	[73]
Alaninyl-d4TMP[a]	+	+	[76]
MTX	1300, 1200	780, 297	[74], [99]
MTX–glucuronide	700	450	[99]
Pemetrexed	281	54	[74]

Table 1 (continued)

Substrates	K_m (μM)	V_{max} (pmoles min^{-} mg^{-} protein)	Reference
Miscellaneous			
Folate	1000	875	[99]
CDCF	12	56	[74]

[a]Substrates used in vesicular transport experiments but K_m and V_{max} not determined for technical reasons
[b]Suggested cotransport

other nuclear receptors in the induction of MRP3 in the liver remains to be demonstrated.

The induction of MRP3 is not restricted to the liver as several bile salts have been shown to induce the expression of *MRP3* in an enterocyte cell line (Caco2) as well as in the murine colon [40].

What is the in vivo role of MRP3?

Based on the basolateral localization of MRP3 in liver and intestine [82, 86], several functions have been proposed for this transporter. The liver and intestine play a key role in the enterohepatic circulation of bile acids as well as in the detoxification of endogenous and exogenous compounds through biotransformation and subsequent excretion. MRP3 was hypothesized to play a role in bile salt physiology because rat Mrp3 transports several conjugated bile salts with high affinity and at considerable rate [38] and hepatic MRP3/Mrp3 is up-regulated during cholestasis [36, 86, 116]. The recent generation of *Mrp3* KO mice has made it possible to study this hypothesis more directly. Two groups independently found that there was no difference in serum levels of bile salts and liver damage between *Mrp3* KO and WT mice after BDL [9, 112], arguing against a prominent role of murine Mrp3 in the basolateral efflux of major bile salts during BDL. This result in mice obviously does not exclude such a role for MRP3/Mrp3 in other species. However, as human MRP3 transports taurocholate with low affinity and at a low rate [109], we expect that MRP3 does not protect the human liver during cholestasis either.

In contrast, glucuronidated hyocholate (HC) and hyodeoxycholate (HDC) are excellent substrates for human MRP3 [112]. These bile salts are formed in humans [75, 83], but not in mice. Liver perfusion experiments with HDC demonstrated that murine Mrp3 significantly contributes to the basolateral excretion of HDC–glucuronide. MRP3 may, therefore, be involved in the excretion of these minor bile salts from the liver.

MRP3 was also thought to play a role in the uptake of bile salts in the ileum and, thereby, to contribute to their enterohepatic circulation. However, *Mrp3* KO mice have no abnormalities in taurocholate uptake in the ileum and total fecal bile salt excretion is similar to that of WT control animals [112]. This demonstrates that there is no role for Mrp3 in the enterohepatic circulation of bile salts, at least in mice. Cholestasis results in increased serum levels not only of bile salts but also of (intrahepatically formed) bilirubin–glucuronides. It is interesting that *Mrp3* KO mice have a diminished ability to excrete bilirubin–glucuronides from the liver into the circulation during BDL, resulting in lower serum bilirubin–glucuronide levels [9, 112]. MRP3 might, therefore, provide an alternative excretion route from the liver when normal canalicular excretion of bilirubin–glucuronide is blocked. Finding other physiological functions of MRP3 in the disposal of endogenous metabolites clearly needs more work. The *Mrp3* KO mice have no overt phenotype and the high concentration of MRP3 in two of the three zones of the adrenal cortex [86] remains unexplained.

Recent papers show, however, that MRP3 has a defense function and contributes to the excretion of toxic organic anions. The preference of MRP3 for glucuronidated compounds combined with its presence in tissues that have a high glucuronidating capacity—like the intestine, kidney and especially the liver—suggested that this transporter has a role in the disposal of xenobiotics and metabolic waste products after their conjugation to glucuronic acid [96]. In agreement with this function is the coordinate induction of MRP3 in the liver along with metabolizing enzymes by several compounds [62, 95]. The transport of acetaminophen– [64], morphine– [111] and hyodeoxycholic acid glucuronide [112] from the liver to the circulation is indeed virtually absent in *Mrp3* KO mice. This results in strongly decreased plasma levels and decreased urinary excretion of these glucuronides. The block in M3G excretion only leads to a modest increase in the concentration of this compound in the liver, as it can be rerouted into the bile. The transporter responsible is MRP2, which is present in the canalicular membrane of the hepatocyte. We have shown that MRP2 can also transport M3G and that in mice lacking Mrp2 excretion into bile is blocked (unpublished observations). Similar results have been obtained with acetaminophen, with mouse Mrp3 [64] and rat Mrp2 [105] mediating transport of acetaminophen–glucuronide from the hepatocyte into circulation and bile, respectively.

These results emphasize the crucial role of MRP2 and MRP3 in the disposal of drug–glucuronide conjugates from the liver, as schematically indicated for morphine in Fig. 1, where Mrp2 and Mrp3 compete for the morphine–glucuronide conjugates in the liver. Note that the affinity of MRP3 for M3G and acetaminophen–glucuronide in vesicular transport assays appears to be low (see Table 1). It is possible that the K_m values determined in vitro do not

reflect the affinity of MRP3 for these substrates in vivo, for instance, because modulatory compounds not present in vitro decrease the K_m in vivo. It is also possible, however, that MRP3 can do its job because MRP3 is a high-capacity pump and because the levels of drug–glucuronide conjugates in liver can reach substantial levels [64, 111].

The induction of MRP3 in the liver can limit the enterohepatic circulation of glucuronidated drugs, resulting in shorter drug exposure. This might result in drug–drug interactions as shown for phenobarbital, which alter the disposal of acetaminophen–glucuronide [90].

MRP3 and multidrug resistance of tumor cells

Whether MRP3 contributes to clinical drug resistance is doubtful. In transfected cells, MRP3 confers low levels of resistance to etoposide [53, 110, 113] and teniposide [53, 110] and higher levels of resistance to MTX in short-time, high-concentration exposures [53]. These low levels of MRP3-mediated resistance might partly be due to the low levels of MRP3 in transfected cells and the presence of other endogenous drug transporters with overlapping drug specificity [110]. One study also found low levels of MRP3-mediated vincristine resistance in transfected HEK293 cells [113]. This result was not confirmed, however, in other cell lines in other laboratories [5, 34, 53]. Several studies have also tried to link *MRP3* expression in cancer cell lines and patient-derived tumor samples to resistance against anti-cancerr agents. The results in cancer cell lines were either negative [32, 52] or the resistance spectrum found did not fit the known drug resistance spectrum associated with MRP3 [106]. MRP3 has been detected in solid as well as in hematological tumors. In pancreatic carcinoma [49] and acute lymphoblastic leukemia [92], tumor grade and survival, respectively, correlated with *MRP3* expression. In contrast, in breast carcinoma samples, *MRP3* expression neither correlated with anthracyclin resistance nor with clinical outcome [32]. Based on the low levels of resistance found in *MRP3*-transfected cells and the narrow spectrum of anti-cancerr agents to which MRP3 mediates resistance, we think that the role of MRP3 in clinically relevant drug resistance will turn out to be limited.

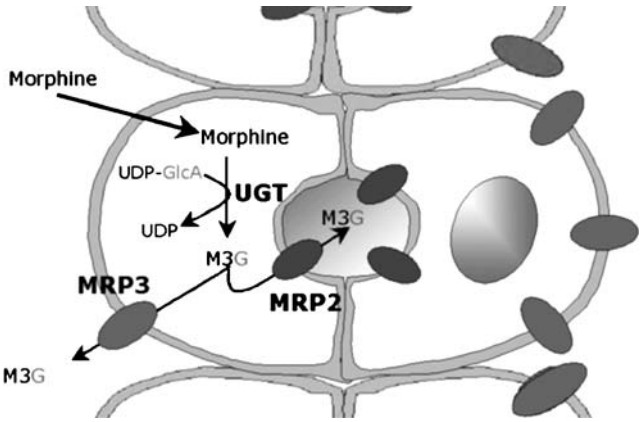

Fig. 1 Schematic representation of the transporters involved in the excretion of morphine-3-glucuronide (M3G) from the murine liver. Morphine that is taken up from the circulation by hepatocytes is converted into M3G, which can be routed into bile by Mrp2 or back to the circulation by Mrp3

MRP4 (ABCC4)

The gene coding for MRP4 was identified in 1996 and is located on the long arm of chromosome 13 at 13q32 [6, 52, 58, 88]. The *MRP4* transcript encodes the smallest of the MRP proteins with a length of 1,325 amino acids with a secondary structure resembling that of MRP5. Both MRP4 and MRP5 lack the additional N-terminal spanning domain that is present in MRP1 [14].

Tissue distribution and expression pattern

The expression of the human *MRP4* gene was initially reported to be restricted to a few tissues [52]. However, subsequent work demonstrated that *MRP4* is widely expressed with mRNA levels ranging from very high in prostate to barely detectable in liver [58]. In fact, a screening of the human expressed sequence tag database against the *MRP4* transcript shows that *MRP4* mRNA is present in almost all tissues except bone marrow, cervix, thymus, vascular endothelium, and soft tissue (http://www.ncbi.nlm.nih.gov). Immunostaining localized the MRP4 protein to the kidney [97], liver [80], erythrocytes [48], adrenal gland [108], platelets [43], brain [67], and pancreas [49] in humans. In rats and mice, *Mrp4* mRNA and protein was also found in many tissues with highest levels in kidney [21, 61, 63]. Mrp4 protein levels in mouse liver are low, and both liver and kidney Mrp4 levels are higher in females than in males [7, 62, 63, 87]. Whether this gender difference also holds for human liver and kidney remains to be investigated.

A unique feature of MRP4 is its dual localization in polarized cells: it can be present apically as well as basolaterally depending on the cell type. Lee et al. [59] localized MRP4 to the basolateral membrane of the tubuloacinar cells of the human prostate by immuno-histochemistry. In contrast, MRP4 was found in the apical membrane in the proximal tubule brush border in the kidney [91, 97]. A basolateral location was also observed in human, rat, and mouse hepatocytes [80] and in pancreatic ductular epithelial cells [49]. In human and murine brain, MRP4 is present in the basolateral membrane of the choroid plexus epithelium and also at the luminal side of capillary endothelium [21, 61, 67]. However, in primary cultured bovine brain microvessel endothelial cells, MRP4 was equally distributed over the apical and basolateral plasma membrane [115]. In conclusion, the location of MRP4 in the membrane is cell and tissue specific, allowing this transporter to be involved in the efflux of substrates into the blood and urine as well as towards adjacent cells into the extracellular matrix or stroma. MRP4 has also been

detected within the membrane of dense delta-granules in human platelets, albeit with only a single polyclonal antibody [43]. The role of MRP4 in these granules remains to be determined.

MRP4 substrate specificity

Schuetz et al. [88] showed that cells selected for resistance against the nucleotide analog, 9-(2-phosphonylmethoxy-ethyl)adenine (PMEA), overproduced MRP4 and subsequent work has established that MRP4 can confer cellular resistance to a range of base, nucleoside, and nucleotide analogs [2, 23, 24, 56, 76, 100]. Cytotoxicity assays performed on cell lines transfected with *MRP4* cDNA constructs have established a wide range of possible substrates amongst anti-cancer and anti-viral compounds (Table 2). The most recent additions to this list are the plant polyphenols resveratrol and quercetin [103]. In the case of the established MRP4 substrates, 6-thioguanine (6TG) and PMEA, drug resistance is due to transport of the nucleoside monophosphate, but not the base, nucleoside, nucleoside diphosphate, or triphosphate [76, 100]. Nucleoside monophosphates are organic anions and, presumably, this is the drug version that is transported also in the case of the other nucleoside/base analogs in the MRP4 resistance spectrum (Table 2).

Cyclic nucleotide transport by MRP4

Multidrug resistant proteins, MRP4, 5, and 8 transport cyclic nucleotides (reviewed in [84]). Although an initial report suggested that MRP4 is a high-affinity cGMP transporter [24], this was not confirmed by later work (see Table 1). An accumulating body of indirect evidence

Table 2 MRP4- and MRP5-mediated drug resistance in transfected cells

Substrate	Resistance factor			
	MRP4		MRP5	
		Ref	Borst lab	Eli Lilly lab
Anti-cancer				
6TG	2.7, 4.1, 4.8	[24], [76], [103]	2.7	8.0
6MP	4.6, 7.5, 5.6	[24], [69], [76]	3.0	ND
5FU			1.0	10.5
5′-deoxy-5-Fluorouridine			ND	2.2
Fludarabine			1.1	1.0
Cytarabine			1.0	5–7
Gemcitabine			1.0	2–3[b]
Cladribine	2.2		1.5	2–7
MTX	5.5[a]	[59]	1.4	4.7
Pemetrexed			ND	7.5
ZD 1694			8.4	ND
GW 1843			2.0	ND
d4T	1.0	[88]		
Irinotecan	5.9	[69]		
SN-38	6	[69]		
Topotecan	1.1	[69]		
Vincristine	1.6	[69]		
Quercetin	2.8	[103]	2.4[c]	
Resveratrol	2.1	[103]	2.3[c]	
Anti-viral				
PMEA	7.3		2.9	ND
bis-POM-PMEA	10.4		3.4	ND
PMEG	3.5			
PMEDAP	9.6			
cPR-PMEDAP	16.6			
Abacavir	2		1.9	ND
Ganciclovir	5	[2]		

References: MRP5 Borst lab: [76], [99], [100], [102], MRP5 Eli Lilly lab: [73]
[a]Short-term exposure, 4 h
[b]Davidson et al. (2002), conference proceeding (Proc. Am. Ass. Cancer Res 43:3868)
[c]Data are taken from [103]

suggests that MRP4 is the main transporter responsible for the efflux of messenger cGMP from erythrocytes [24, 48, 85, 89, 93, 104]. However, all experiments were conducted in vitro with inside-out membrane vesicles prepared from either cultured cell lines or erythrocytes. cGMP efflux from intact erythrocytes has not yet been demonstrated and there is no evidence that mature erythrocytes can actually make cGMP. The physiological relevance of cGMP efflux by MRP4 from erythrocytes is, therefore, questionable, especially as MRP4 transports cGMP with low affinity [48, 85, 101]. In view of the observed high rate of cGMP transport by erythrocyte vesicles, MRP4 might limit the ability of erythrocytes to accumulate base/nucleoside analogs. Erythrocytes may function as a carrier system in the transport of endogenous compounds and xenobiotics, such as anti-cancer agents 6-mercaptopurine (6MP) and 6TG, through the body. Active low affinity, high capacity, and efflux of these compounds and their metabolites from the erythrocyte by MRP4 might, therefore, limit the bioavailability of these drugs [31].

MRP4 is present at high levels in the apical membrane in human kidney brush border proximal tubules [97] and is, therefore, a candidate for the active secretion of cGMP and cAMP in urine. However, a recent publication by Chen et al. [22] shows a twofold increase of Mrp4 protein in kidneys of Mrp2-deficient TR^- rats without the expected increase in excreted cAMP (cGMP was not measured). Moreover, another group reported that the down-regulation of Mrp4 in rat kidney under cholestatic conditions coincides with an increase in secreted cAMP in urine [29]. These two studies do not support an important role for renal Mrp4 in the excretion of urinary nucleotides, even though the authors postulate that Mrp4 is, in fact, the main cAMP transporter. The *Mrp4* KO mouse [61] might help to clarify this issue.

What is the function of MRP4?

Vesicular uptake experiments (summarized in Table 1) with inside-out vesicles prepared from cells transfected with *MRP4* cDNA constructs have identified two potential physiological MRP4 substrates: bile salts [79, 80] and urate [98]. The transport of the bile salts taurocholate, cholate, and glycocholate was dependent on the presence of GSH (analogs). Such dependence has not been observed for other MRP4 substrates [48, 77, 101, 108]. Given the relatively low rates of bile salt and urate transport observed, it would be important to verify the postulated role of MRP4 in the transport of these substrates in *Mrp4* KO mice.

Although hepatic MRP4 protein levels are relatively low, they increase in mice and rats under hepatic stress [3, 4, 29]. This was first seen in *Fxr* KO mice, which have reduced levels of the major canalicular bile salt pump, BSEP, and increased hepatic *Mrp4* mRNA [87]. In all models, the plasma and urine levels of bile salts and/or cAMP were elevated. In contrast, the Mrp4 levels in the livers of Mrp2-deficient rats are not elevated [22, 44]. Acute hepatotoxic stress and hyperbilirubinemia due to Mrp2 deficiency obviously activate different compensatory routes.

A class of endogenous substrates to MRP4 that is structurally related to the bile salts is represented by conjugated steroids such as $E_2$17βG [24] and dehydroepiandrosterone-3-sulphate (DHEAS) [108]. A high-affinity transport of prostaglandin E_1 (PGE_1) and PGE_2 [77] and other prostanoids [81] was also demonstrated. Whether the transport of conjugated steroids and prostanoids by MRP4 is physiologically important remains to be determined.

In human and murine brain, MRP4 is mainly present in the capillary endothelium, but MRP4 has also been detected in the basolateral membrane of the choroid plexus epithelium and in astrocytes [21, 61, 67]. These locations of MRP4 indicate that it may contribute to the barrier function at the blood–brain barrier (apically in capillary endothelium) and at the blood–cerebrospinal fluid barrier (basolaterally in choroid plexus epithelium). An initial report which investigates the putative barrier function for MRP4 seems to confirm this hypothesis; levels of the anti-cancerr agent topotecan in brains from *Mrp4* KO mice were higher than in those from WT mice [61]. The presence of MRP4 in the astrocytic membrane implies that MRP4 may also be involved in the transport of endogenous substrates in brain (e.g., cGMP, cAMP, and GSH). The role for MRP4 in the efflux of drugs and other compounds from the brain and its importance in the efflux of cyclic nucleotides and GSH from astrocytes and possibly other cells in the brain remain to be investigated.

MRP4 and multidrug resistance of tumor cells

Although MRP4 is present in many human cancer cell lines [52], there is no conclusive evidence linking MRP4 to drug resistance in human tumors. To date, only a single report by Norris et al. [69] suggests a correlation between increased MRP4 levels and tumor prognosis in patients with primary neuroblastoma. The authors attribute this correlation to the ability of MRP4 to transport irinotecan and its active metabolite SN38 (Table 2).

MRP5 (ABCC5)

Initial studies

Early work on MRP5, summarized by [13, 15, 16, 35], showed that MRP5 is a typical organic anion pump transporting acidic organic dyes [65] (but not calcein), DNP–GS, and GSH [102] and inhibited by sulfinpyrazone and benzbromarone [102]. Like MRP4 and MRP8, MRP5 stands out through its ability to transport nucleoside monophosphate analogs [102]. Where tested, neither the corresponding nucleoside nor the nucleoside di- and triphosphates are transported.

An analysis of tissue RNA indicated that *MRP5* is ubiquitously expressed, albeit at a low level, as the available antibodies only barely detected the protein in

tissues. In transfected epithelial cells, MRP5 routes to the basolateral membrane [102].

Cyclic nucleotide transport by MRP5

Jedlitschky et al. [42] discovered that MRP5 transports the cyclic nucleotides 3′, 5′-cAMP and -cGMP. In transport experiments with vesicles of membranes derived from V79 hamster lung fibroblasts transfected with a human *MRP5* cDNA construct, they obtained K_m values of 2.1 μM for cGMP and 379 μM for cAMP. Interestingly, cGMP transport was highly sensitive to inhibitors of cGMP phosphodiesterase, such as zaprinast, trequinsin, and sildenafil. Trequinsin was found to inhibit cGMP transport competitively with a K_i of 240 nM.

Although other labs have confirmed the ability of MRP5 to transport cGMP, they obtained a much lower affinity for this substrate. cGMP was found to compete with other MRP5 substrates for transport, but half-maximal inhibition required concentrations of about 1 mM, not in micromolars [74, 76]. Low-affinity MRP5-mediated efflux of cyclic nucleotides was also observed from intact HEK cells in which intra-cellular cGMP production was stimulated with nitroprusside or cAMP production with forskolin [101]. The authors conclude "that MRP5 is a low-affinity cyclic nucleotide transporter that may at best function as overflow pump, decreasing steep increases in cGMP levels under conditions where cGMP synthesis is strongly induced and phosphodiesterase activity is limiting" [101].

The high sensitivity of MRP5 to cGMP phosphodiesterase inhibitors, reported by Jedlitschky et al. [42], has not been confirmed in other labs either. In vesicular transport experiments with alaninyl-d4TMP as substrate, Reid et al. [76] saw only marginal inhibition by 1 μM zaprinast and no inhibition by 1 μM sildenafil or trequinsin. Half-maximal inhibition of 5-FdUMP transport by these drugs required 20 μM or more [74].

It is unlikely that the discrepancy between the results of Jedlitschky et al. [42] and of other labs [74, 76, 101] is due to *MRP5* polymorphisms. The cDNA construct used in [42] was kindly provided to us by Dr. Jedlitschky. In intact HEK293 cells, this construct produced MRP5 able to extrude cGMP from the cells, but only with low affinity (I. van der Heijden and P. Borst, unpublished). We think that the high-affinity, sildenafil-sensitive cGMP transport observed in V79 hamster cells by Jedlitschky et al. [42] may have been due to up-regulation of an endogenous transporter. As these cells are not available to other investigators, this conjecture [101] cannot be verified.

Transport of nucleotide analogs by MRP5

One of the characteristic properties of MRP5, which it shares with MRP4 and MRP8, is its ability to transport nucleoside monophosphate analogs. Table 2 summarizes the data available for resistance of *MRP5*-transfected cells against the analogs. Table 1 presents the results of published vesicular transport experiments. A problem in presenting these results is that the two labs in which these experiments were done get different results, notwithstanding the use of *MRP5* constructs with identical sequences introduced in the same cell type, HEK293. A reasonable explanation for the discrepancy is that the single transfectant studied in the Eli Lilly lab has more functional MRP5 in the cellular plasma membrane than the 5I clone used for most experiments in the Borst lab. Clones with a high level of MRP5 in the plasma membrane are not easily obtained, as a major unpublished screening effort in the Borst lab has failed to turn up *MRP5* transfectants with resistance levels as high as those of clone 36 of the Lilly group [74].

Vesicular transport experiments with MRP5 made a sluggish start because none of the standard MRP substrates worked. Reid et al. [76] (Table 1) then found transport of alaninyl-d4TMP, but this was not a convenient substrate because so little was available that no K_m or V_{max} could be determined. Nevertheless, this substrate was useful for competition experiments, which showed that MRP5 has a very low affinity for the nucleotide analogs that it transports (Table 3) [76], confirming earlier conclusions from experiments with intact cells [100]. Several other useful substrates that are readily available were found more recently (Table 1), notably MTX and the dye dicarboxydichlorofluorescein diacetate (CDCF) [73]. The only substrate with micromolar affinity is CDCF. The affinity of MRP5 for nucleotide analogs, for MTX and its analogs, and for folate is low.

The only "natural" nucleoside monophosphate that interacts with MRP5 is dUMP (Table 3), although with a very low affinity. The inhibition by 5-FudR (Table 3) is also unusual and the only example of a nucleoside analog interacting directly with MRP5 rather than after conversion into a nucleotide [74, 76, 100, 102]. Note also in Table 1 that MRP5 is able to transport MTX–glucuronide, a talent it shares with BCRP (ABCG2); MRP1–4 transport MTX, but not MTX–glucuronide [99].

Table 3 Inhibition of MRP5-mediated transport by nucleotide and MTX analogs

Compound (mM)	Half-maximal inhibition of transport of		
	Ala-d4TMP (1μM)	5-FdUMP (0.25mM)	CDCF (5μM)
MTX	–	0.8	3.0
5-FdUMP	–	1.7	2.5
dUMP	–	9.5	ND
5-FudR	–	0.5	1.5
Pemetrexed	–	2.1	0.4
PMEA	>1	–	–
Cytarabine MP	>1	–	–
Fludarabine	>1	–	–
Gemcitabine MP	>1	–	–

Data are taken from [73], [74], and [76]

Miscellaneous substrates

Long lists of compounds that appear not to be transported by MRP5 have been assembled [65, 74, 76, 102]. McAleer et al. [65] reported weak (about threefold) resistance of *MRP5* transfectants against cadmium chloride and antimonyl tartrate (but not arsenite, which usually accompanies antimonite resistance), but this has not been confirmed in a later work. McAleer et al. [65] also found reduced uptake of fluorochromes, such as 5-chloromethylfluorescein diacetate, in *MRP5* cells and this has recently been confirmed and extended by Pratt et al. [73], who showed that the closely related CDCF, which is membrane impermeable, is suitable for vesicular transport studies (Table 1).

Pratt et al. [74] find for their *MRP5* transfectant a threefold resistance to cisplatin and oxaliplatin and a twofold resistance to doxorubicin, but no cisplation or doxorubicin resistance was observed by McAleer et al. [65] and Wijnholds et al. [102]. Wijnholds et al. [102] conversely found some resistance to etoposide/teniposide not found elsewhere [102]. It is unlikely that MRP5 will obviously contribute to MDR or platin drug resistance in the clinic.

Inhibitors of MRP5

Table 4 summarizes results obtained with intact cells and in vesicular transport experiments. MRP5 is inhibited by the standard organic anion transport inhibitors such as benzbromarone, probenecid, sulfinpyrazone, and MK571, but only at fairly high concentrations. The only compound inhibiting at relatively low concentrations is NPPB, known as a cystic fibrosis transmembrane conductance regulator inhibitor. Unfortunately, none of the compounds in Table 4 qualifies as a specific MRP5 inhibitor.

Some of these compounds were also shown to inhibit cGMP efflux from *MRP5*–HEK293 cells [101]. The dyes rose Bengal and indocyanine green inhibited >80% at 100 μM and also inhibited MRP4 [101].

The tissue distribution of MRP5

Early studies on MRP5 found *MRP5* RNA in all tissues and cell lines tested, but at relatively low levels [52, 102]. Getting more detailed information on the tissue localization of the MRP5 protein has proved to be difficult as an increasingly numerous army of monoclonal antibodies failed to give clear pictures. Even the *Mrp5* KO mouse, which provides the ideal negative control, has not been helpful (P. Wielinga, J. Wijnholds, and P. Borst, unpublished).

Additional studies showed the presence of *MRP5* RNA in many cell lines [72, 94] and in macrophages [45]. Functional assays have shown export of PMEA from cultured rat microglial cells containing both Mrp4 and 5 [27]. The only MRP5 tissue distribution studies were done by Jedlitschky and co-workers with a single polyclonal antibody directed against the C-terminal 14 amino acids of MRP5 [68]. This antibody detects MRP5 in smooth muscle cells of the human genito-urinary tract and in epithelial cells of ureter and urethra and in blood vessels [68]. In placenta, MRP5 was found in the basal membrane of syncytiotrophoblasts and in and around fetal vessels [66]. In heart, MRP5 was found in cardiomyocytes, endothelial cells, and smooth muscle cell [28]. In the brain, MRP5 was found in astrocytes and in pyramidal neurons. Remarkably, MRP5 was also found at the luminal (apical) side of brain capillary endothelial cells, even though it routes basolaterally in MDCK cells [102]. Whether MRP5 is in other human epithelia is not clear from the papers of Jedlitschky et al. [28, 66–68].

Table 4 Inhibitors of MRP5-mediated transport

Compound	Inhibition of transport of			
	PMEA	5FdUMP (250μM)	CDCF (5μM)	MTX (100μM)
	IC50 (μM)	EC50 (μM)	EC50 (μM)	EC50 (μM)
Benzbromarone	150	–	–	<100
Probenecid	200	70	34	<100
Sulfinpyrazone	300	–	103	–
MK571	40	17	16	<100
NBMR	>100	–	–	–
Dipyridamole	30	–	–	100
Sildenafil	80	600	–	>100
Trequinsin	30	600	219	>100
Zaprinast	250	20	32	<100
Glibenclamide	–	15	105	–
NPPB	–	2	0.1	–

Data are taken from [73], [74], and [76].

What is the function of MRP5?

The discovery by Jedlitschky et al. [42] that MRP5 transports cGMP with high affinity seemed to provide an appealing function for MRP5, as cells are known to export cGMP under some conditions. This cGMP export function has been elaborated on in later papers by Jedlitschky et al. [28, 66–68] but remains controversial as the high-affinity cGMP transport by MRP5 has not been confirmed in other labs. The possibility that MRP5 contributes to resistance against nucleotide analogs [73, 74, 76, 102] or MTX (analogs) [73, 99] remains open, but, also for these compounds, the affinity of MRP5 is low. The *Mrp5* KO mouse and even the *Mrp4/5* double KO mouse has no spontaneous phenotype (P. Wielinga, J. Schuetz, J. Wijnholds, and P.Borst, unpublished). For the moment, we have to admit that the function of MRP5 remains unknown and that even plausible conjectures are not available.

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