Review

Multidrug resistance associated proteins in multidrug resistance

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Abstract

Multidrug resistance proteins (MRPs) are members of the C family of a group of proteins named ATP-binding cassette (ABC) transporters. These ABC transporters together form the largest branch of proteins within the human body. The MRP family comprises of 13 members, of which MRP1 to MRP9 are the major transporters indicated to cause multidrug resistance in tumor cells by extruding anticancer drugs out of the cell. They are mainly lipophilic anionic transporters and are reported to transport free or conjugates of glutathione (GSH), glucuronate, or sulphate. In addition, MRP1 to MRP3 can transport neutral organic drugs in free form in the presence of free GSH. Collectively, MRPs can transport drugs that differ structurally and mechanistically, including natural anticancer drugs, nucleoside analogs, antimetabolites, and tyrosine kinase inhibitors. Many of these MRPs transport physiologically important anions such as leukotriene C4, bilirubin glucuronide, and cyclic nucleotides. This review focuses mainly on the physiological functions, cellular resistance characteristics, and probable *in vivo* role of MRP1 to MRP9.

Key words Multidrug resistance protein (MRP), multidrug resistance (MDR), ABC transporter, chemotherapy

Chemotherapy is one of the major treatment modalities available for cancer patients. Unfortunately, during the course of treatment, cancer cells develop resistance to functionally and structurally different anticancer drugs by either acquired (due to host factors) or intrinsic (due to genetic or epigenetic) mechanisms[1,2]. This phenomenon of resistance to different classes of anticancer drugs by cancer cells is termed multidrug resistance (MDR). This pervasive and insidious clinical problem eventually leads to cancer relapse and death among patients. The mechanisms of MDR have been intensively studied, although not all mechanisms producing MDR have been elucidated. The detailed mechanisms that cancer cells utilize or develop to evade chemotherapy are complex and have been described in detail in several recent reviews[3-5]. One of the most important mechanisms underlying MDR is overexpression of adenosine triphosphate (ATP)–binding cassette (ABC) transporters, which efflux a wide spectrum of anticancer drugs against the concentration gradient using ATP-driven energy.

The ABC transporter family, representing the largest family of transmembrane proteins, comprises 49 transporters that are further subdivided into seven subfamilies, ABC-A to -G, based on sequence similarities[6]. Of them the major ABC transporters involved in MDR development are ABC subfamily B member 1 [(ABCB1/P-glycoprotein (P-gp)], ABC subfamily G member 2 [ABCG2, also known as breast cancer resistance protein (BCRP)/mitoxantrone resistance protein (MXR)/placenta-specific ABC protein (ABCP)], and ABC subfamily C member 1 (ABCC1/MRP1)[6,7]. This review will provide in-depth details about the MRPs involved in conferring MDR in cancer cells.

The MRP subfamily, the C subset of the ABC transporter superfamily, is composed of thirteen members, and nine of these are primarily involved in MDR (Table 1)[8]. Based on functional characterization, localization, and cloning studies, these nine MRPs have been established as ATP-dependent efflux transporters for endogenous substances and xenobiotics. The other three members of the MRP subfamily, namely ABCC7/cystic fibrosis transmembrane conductance regulator (CFTR), ABCC8/sufonylurea receptor 1

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Table 1. Summary of MRP members involved in MDR

MRP member	Alternative name[188]	Amino acid identity with MRP1 (%)[63]	Physiological substrate[63]	Tissue distribution[188]
MRP1	ABCC1	100	LTC_4, E_1S, $E_217\beta G$, folate	Ubiquitous
MRP2	ABCC2, cMOAT, cMRP	50	LTC_4, E_1S, $E_217\beta G$	Liver, kidney, gut
MRP3	ABCC3, MOAT-D, cMOAT-2	58	LTC_4, $E_217\beta G$, cholylglycine	Liver, adrenals, pancreas, kidney, gut
MRP4	ABCC4, MOAT-B	41	cAMP, cGMP, LTC_4, PGE_2, folate, urate	Prostate, lung, muscle, pancreas, testis, ovary, bladder, gallbladder
MRP5	ABCC5, MOAT-C, pABC11	38	cAMP, cGMP, folate, 2′-deoxyuridine 5′-monophosphate	Ubiquitous
MRP6	ABCC6, MOAT-E, MLP-1, ARA	46	LTC_4, S-glutathionyl N-ethylmaleimide	Liver, kidney
MRP7	ABCC10	35	LTC_4, $E_217\beta G$	Pancreas, testis, colon, spinal cord, tonsils, lung, trachea, skin
MRP8	ABCC11	33	DHEAS, LTC4, E217βG, cAMP, cGMP, cholylglycine, folate	Breast, ovary, lung, testis, kidney, liver, colon, and brain
MRP9	ABCC12	36	?	Breast, testis, brain, skeletal muscle, ovary

MRP, multidrug resistance protein; MDR, multidrug resistance; MLP-1, MRP-like protein 1; ARA, anthracycline resistance associated. The question mark (?) indicates that information is not available.

(SUR1), and ABCC9/SUR2, are not involved in conferring MDR. ABCC7 is a regulated chloride channel, whereas ABCC8 and ABCC9 are intracellular ATP sensors and regulate the specific K^+ channel permeability[9]. On the basis of structural topology, the nine main MRPs can be divided into two groups. One has a typical ABC transporter structure and is composed of two membrane spanning domains (MSD) with nucleotide binding domains (NBD1 and NBD2) in between (Figure 1). These can be referred to as "short MRPs" and include MRP4, MRP5, MRP8, and MRP9 (ABCC4, 5, 11 and 13, respectively). The other group, which includes MRP1, 2, 3, 6 and 7 (ABCC1, 2, 3, 6 and 7, respectively), have an additional MSD (MSD_0) and are referred as "long MRPs"[10-12].

MRP1/ABCC1

MRP1 was first discovered in an anthracycline-resistant cell line HL60/Adr which was the first major ABC transporter other than P-gp. This protein was shown to have a molecular weight of 190 kDa[13-16], and Cole *et al.*[10] subsequently isolated the cDNA from a human lung cancer H69AR cell line. The protein was named multidrug resistance protein 1 (MRP1). Further studies showed MRP1 was present on the basolateral surface of the epithelial membrane and was involved in ATP-dependent efflux of xenobiotics across the cell membrane[17,18] (Figure 1).

MRP1 is reported to be widely expressed in various tissues including lung, testis, kidney, skeletal and cardiac muscles, placenta, and macrophages[11,19]. It has also been found to be predominantly localized to blood-tissue barriers, such as the basolateral membrane of the choroid plexus cells of the blood-cerebrospinal fluid barrier, the bronchial epithelium[20,21], and the apical syncytiotrophoblast membrane of the placenta[22]. MRP1 and P-gp share only 15% amino acid sequence identity and posses some distinct features. Structurally, MRP1 differs from P-gp in that it has an extra MSD, named MSD_0, with five transmembrane (TM) helices, and two other MSDs, each having six TM helices with two NBDs in between (Figure 1)[23,24]. MSD_0 does not play a role in trafficking to the plasma membrane or efflux activity, but it is required for efficient retention of MRP1 at the cell surface[17].

In spite of the modest degree of amino acid sequence identity with P-gp, MRP1 has a significant overlapping resistance profile with P-gp. The resistance profile, characterized with the help of transfected cell lines, established that MRP1 confers resistance to a wide range of anticancer drugs such as anthracyclines, vinca alkaloids, epipodophyllotoxins, camptothecins, methotrexate (MTX), saquinavir, and mitoxantrone (MX); however, distinct from P-gp, it does not confer resistance to taxanes, an important component of the P-gp resistance profile (Table 2)[25-30]. Studies with fibroblast cell lines from *Mrp1* knockout mice show a similar resistance pattern[31-33], along with modest sensitization to taxanes and MX. So far, miniscule data are available regarding the involvement of MRP1 in conferring resistance against taxanes and MX. Some newer classes of targeted anticancer drugs, such as tyrosine kinase inhibitors (TKIs, e.g. imatinib), also succumb to

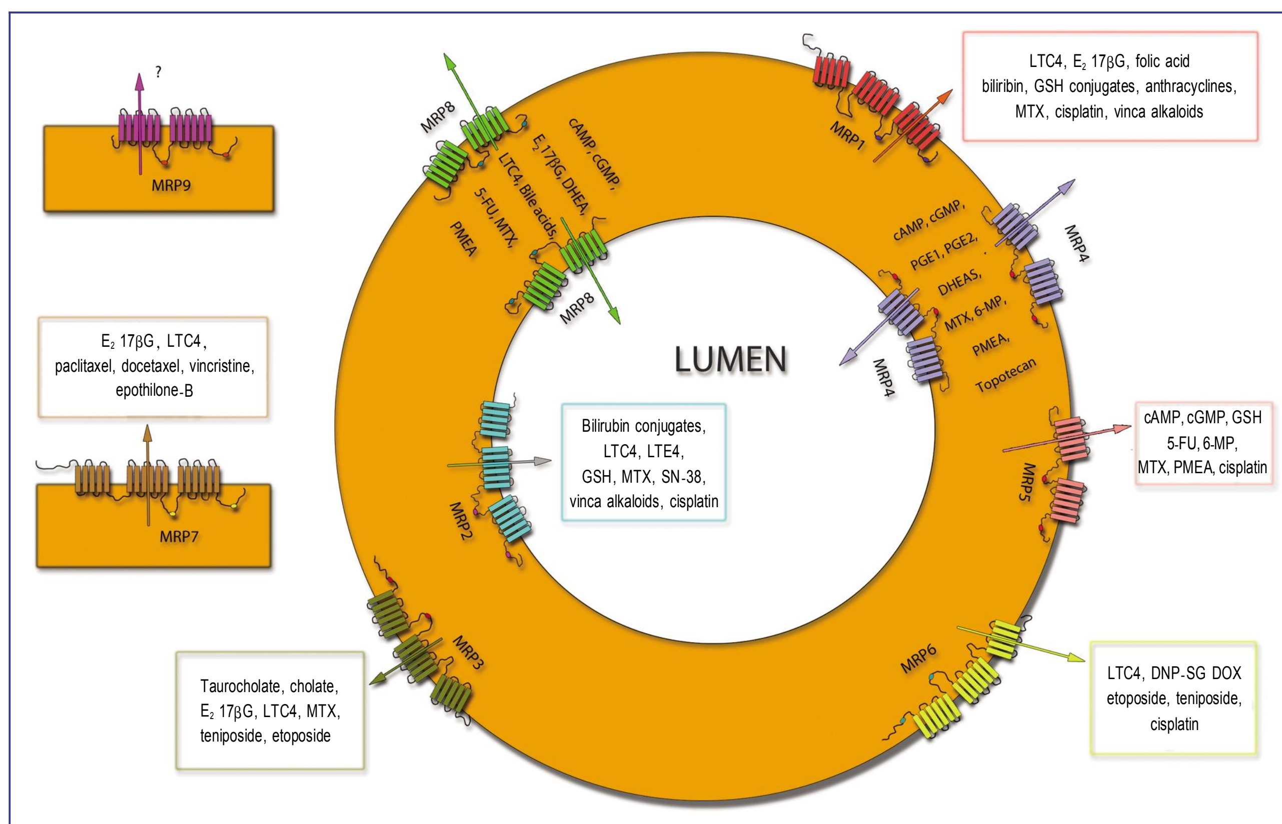

Figure 1. **The pictorial depiction shows the topography and the location of both the short- (MRP4, MRP5, MRP6, MRP8 and MRP9) and long-form (MRP1, MRP2, MRP3, and MRP7) members of the MRP subfamily.** Each of these transporters pumps out a variety of endogenous and xenobiotic substrates. These transporters are mainly present either apically or basolateraly; however, the localization of MRP7 and MRP9 is still unknown. The substrates of MRP9, the last member to be cloned among the MRP subfamily, are still unknown and are open for discussion. MRP, multidrug resistance protein.

MRP1-mediated resistance[33].

Though MRP1 has an overlapping resistance profile with P-gp, the physiological substrate profile differs significantly. While substrates for P-gp are neutral or mildly positive lipophilic compounds, membrane vesicle transport studies established MRP1 as a lipophilic anionic transporter that can transport glutathione conjugates, such as leukotriene C4 (LTC4) and dinitrophenyl-S-glutathione (DNP-SG)[34,35]. It can also transport glucuronate conjugates (e.g., $E_2$17β G), dianionic bile salts, and sulfate conjugates[36-38]. A study using an *Mrp1* knockout mouse model also confirmed that LTC4 is indeed a physiological substrate of MRP1 (Table 1)[38].

MRP1 is a basolateral transporter whose activity results in the movement of compounds into tissues that lie beneath the basement membrane[39]. Transport of glutathione and glucuronate conjugates by MRP1 is of interest because they represent phase II metabolism and cellular detoxification. Efflux pumps involved in cellular export have been referred to as GS-X pumps in the case of glutathione (GSH) conjugates[40], and MRP1 has widespread expression and glutathione conjugate efflux characteristic, which indicates MRP1 as GS-X pump[41]. This feature of MRP1 explains the transport capacity of MRP1 for MTX, an organic anion, and arsenite, which can form complex with GSH molecules[42]. In addition, vinca alkaloids and anthracyclines, to which MRP1 confers resistance, are weak organic bases and do not conjugate with acidic ligands in human cells. Hence, resistance to these compounds by MRP1 was unclear. However, recent studies indicate that these drugs are probably co-transported with GSH and that cellular depletion of GSH decreases MRP1-mediated resistance to these drugs. In addition, similar results have been reported in vesicular transport assays of vincristine and daunorubicin[4,42-46]. The detailed transport mechanism for GSH by MRP1 has been postulated and reviewed by Kruh *et al.*[18].

Clinically, MRP1 levels are elevated in numerous cancer types, such as non–small cell lung cancer (NSCLC)[20,47], breast cancer, and prostate cancer[47], and they are also related to accelerated relapse in breast cancer[48]. MRP1 expression has been reported in several

Table 2. Summary of MRP members involved in MDR

Anticancer drugs		MRP1	MRP2	MRP3	MRP4	MRP5	MRP6	MRP7	MRP8	MRP9
Antimetabolites	6-mercaptopurine	–	–	–	+	+	–	–	–	?
	6-thioguanine	–	–	–	+	+	–	–	–	?
	5-fluorouracil	–	–	–	–	+	–	–	+	?
	Methotrexate	+	+	+	+	+	–	–	–	?
Antibiotics	Daunorubicine	+	–	–	–	–	+	–	–	?
	Doxorubicine	+	+	–	–	–	+	–	–	?
	Epirubicine	+	+	–	–	–	+	–	–	?
	Actinomycine D	–	–	–	–	–	+	–	–	?
	Mitoxantrone	–	+	–	–	–	–	–	–	?
Platinum drug	Cisplatin	–	+	–	–	+	+	–	–	?
Taxanes	Paclitaxel	–	+	–	–	–	–	+	–	?
	Docetaxel	–	+	–	–	–	–	+	–	?
Vinca alkaloids	Vincristine	+	+	–	–	–	–	+	–	?
	Vinblastine	+	+	–	–	–	–	+	–	?
Epipodophyllotoxins	Etoposide	+	+	+	–	–	+	–	–	?
	Teniposide	–	–	+	–	–	+	–	–	?
Camptothecins	Irinotecan	+	–	–	+	–	–	–	–	?
	Topotecan	–	+	–	+	–	–	–	–	?
	SN-38	+	–	–	+	–	–	–	–	?
Tyrosine kinase inhibitors	Imatinib	+	–	–	–	–	–	–	–	?
	Gefitinib	+	–	–	–	–	–	–	–	?
Miscellaneous	Epothilone B	–	–	–	–	–	–	+	–	?

Adapted from Tiwari *et al.*[49]. "+" indicates that the drug is a substrate for the particular ABC transporter. "–" indicates that the drug is not a substrate for the particular ABC transporter. Other footnotes as in Table 1.

solid and hematological cancers. Negative correlation between MRP1 expression and response to treatment has also been found. Such studies have been reviewed in detail elsewhere[11,18,49,50]. However, there is no definite consensus drawn with respect to the role of MRP1 in acquired resistance or in prognosis.

MRP2/ABCC2

Mrp2, the second member of the MRP subfamily of ABC transporter, was first cloned from rat hepatocyte and was named as a hepatocellular canalicular multiple organic anion transporter (cMOAT)[51]. MRP2 shares 49% amino acid identity with MRP1 but it has a different expression pattern. While MRP1 is widely expressed in many tissues, MRP2 is mainly expressed in the apical (canalicular) hepatocyte plasma membrane, small intestine, and renal proximal tubules (Table 1)[52-54]. *MRP2* mRNA is present in the peripheral nerves, gallbladder, placental trophoblasts, and CD4$^+$ lymphocytes[22,55,56].

Because MRP2 handles a range of conjugates similar to that of MRP1, it was believed to confer resistance to similar anticancer drugs as well. This hypothesis was formulated based on an experiment in which an antisense *MRP2* RNA construct was introduced into human hepatocellular carcinoma HepG2 cells, resulting in enhanced sensitivity to several anticancer drugs such as cisplatin, vincristine, doxorubicin, and the camptothecin derivatives CPT-11 and SN-38[57]. Evers *et al.*[58] later showed that MRP2 could transport vinblastine in polarized Madin Darby canine kidney epithelial (MDCK) cells, suggesting a potential role for MRP2 in vinblastine resistance. In addition, *MRP2*-transfected cells also conferred resistance to MTX[59], cisplatin, etoposide, doxorubicin, and epirubicin[60]. The resistance capacity of MRP2 to cisplatin is quite interesting because MRP1 does not confer resistance to cisplatin (Table 2)[25,27]. However, this phenomenon is convincible, as cisplatin is well known to form toxic GSH complexes in the cells[61].

MRP2 and MRP1 have very similar substrate specificities and mediate transport of some hydrophobic compounds in the presence of GSH, though with different affinities[62]. MRP2 has a transport facility for organic anions including sulfate, glucuronide, and GSH conjugates (Table 1)[63-65]. Furthermore, MRP2 is involved in the biliary elimination of certain endogenous conjugates, such as LTC4 and conjugated bilirubins[63,65]. Though these conjugated metabolic complexes are thought to be detoxified, their accumulation may result in reformation of active compounds either spontaneously or by enzymatic hydrolysis.

Mutations within human *MRP2* result in an inactive MRP2 protein in the canalicular membrane as observed in Dubin-Johnson syndrome (DJS), a hereditary disorder

with modest elevation of serum conjugated albumin[52,66,67]. Eisai hyperbilirubinuria rats (EHBRs) and Groninger Yellow transporter rat strains are deficient in *Mrp2* and are perfect models to study human DJS[67-69].

MRP2 expression has been reported in several human tumor cell lines of lung, gastric, renal, and colorectal cancers[70]. Moreover, few cisplatin- and doxorubicin-resistant cell lines have shown overexpression of *MRP2* mRNA[55,71]. Recent reports by Korita *et al.*[72] suggest that efficacy of cisplatin-based chemotherapy in patients with hepatocellular carcinoma depends upon MRP2 expression level.

MRP3/ABCC3

The MRP3 protein localizes in the basolateral membrane domain of polarized cells. It was first identified in human and rat hepatocytes, mediating efflux of organic anions into sinusoidal blood. Among the MRPs with known coding sequences, MRP3 shares the highest degree of structural similarity with MRP1 (58% amino acid identity)[73,74]. MRP3 expression is found in adrenal glands, kidney, small intestine, colon, pancreas, and gallbladder, and with a lower expression in the lungs, spleen, stomach, and tonsils[55,75-77].

Although MRP3 has high structural similarity with MRP1, the affinity of MRP3 for conjugates is considerably lower than that of MRP1. Its drug resistance capabilities are also as extensive as neither MRP1 nor MRP2. The narrow and limited drug resistance profile of MRP3 to epipodophyllotoxins, vincristine, and MTX was reported previously in studies using *MRP3*-transfected cells [74,78]. Resistance potency to etoposide and vincristine is quite low compared to MRP1 (Table 2). In contrast to MRP1 and MRP2, MRP3 does not need GSH to transport natural products [79]. In addition, Kool *et al.*[74] reported an unchanged level of GSH in *MRP3*-transfected cells. These findings may explain why MRP3 has limited resistance properties—because it has a lower affinity for amphipathic anions and GSH. In recent reports, significant accumulation of etoposide glucuronide in the liver in *Mrp2*$^{-/-}$/*Mrp3*$^{-/-}$ mice was described, but neither single knockout showed this phenomenon, indicating an alternative pathway provided by Mrp2 and Mrp3 for hepatic elimination of etoposide glucuronide[80].

Elevated Mrp3 expression has been reported in cholestatic rat liver[69,75] and cholestatic human liver[81], as well as in patients with DJS who lack functional MRP2 in the liver canalicular membranes. This suggests that basolateral MRP3 expression in hepatocytes may allow efflux of organic anions from the liver into the blood upon blockade of bile secretion, and that MRP3 is a back-up system for amphipathic anions in cholestatic conditions. Another study revealed Mrp3 as an alternative exporter of bile acids and glucuronides from cholestatic hepatocytes, but the pump was not involved in the enterohepatic circulation of bile acids in *Mrp3* knockout mice models[82]. Membrane vesicles, prepared from *MRP3*-transfected HEK293 cells, were reported to transport LTC4, DNP-SG, and $E_2 17\beta$ G, prototypical MRP1 substrates, with low affinity[83]. MRP3 confers resistance to and transport capacity for MTX[74,84]. Increased expression of MRP3 has been reported in human hepatocellular carcinomas[85], primary ovarian cancer[86], and adult acute lymphoblastic leukemia (ALL)[87].

MRP4/ABCC4

MRP4, the fourth member of the MRP subfamily of ABC transporters[88,89], is one of the shortest members, encoding 1325 amino acids. The gene was discovered in 1996 in a T-lymphoid cell line[74], and it is located on chromosome 13q32.1[55,88,90-92]. Structurally, MRP4 is composed of a typical ABC transporter core consisting of two NBDs and two MSDs, each MSD with six TMDs (Figure 1)[11,93,94]. In addition, the TM6 subunit of MRP4 was found to be conserved among all species[11].

With its dual localizations in the apical (the renal proximal tubule cells and the luminal side of brain capillary endothelium) and basolateral membranes (the prostate tubuloacinar cells, hepatocytes, and choroid plexus epithelium), MRP4 differs from other MRPs that are either located apically or basolaterally[95]. A sequence-based tag against human *MRP4* transcript revealed that *MRP4* mRNA is present in all tissues except the bone marrow, thymus, vascular endothelium, and soft tissues[93]. MRP4 can pump out diverse endogenous and xenobiotic organic anionic compounds along with their phase II metabloites, thereby conferring resistance to various cytotoxic compounds and, in turn, protecting crucial tissues against them[11,93].

MRP4 has a wide range of substrate specificity, including antiviral (adefovir, tenofovir, ganciclovir), antibiotic (chephalosporins), cardiovascular (loop diuretics, thiazides, angiotensin II receptor antagonists), and cytotoxic drugs [MTX, 6-thioguanine (6-TG), 6-mercaptopurine (6-MP), topotecan][96-98]. Cancer cells selected with a nucleotide analog, 9-(2-phosphonylmethoxy-ethyl) adenine (PMEA), were found to overexpress MRP4 (Table 2)[89]. Subsequently, MRP4 was found to confer resistance to a wide range of base, nucleotide, and nucleoside analogs[99-104]. Plant polyphenols, resveratrol, and quercetin are newer additions to the list of substrates for this MRP[105]. In addition, it was seen that with the established nucleoside substrates of MRP4, such as 6-TG and PMEA, only the monophosphate form of the nucleoside analogues and not the diphosphate or triphosphate forms are transported by the pump. This could be due to the fact that

nucleoside monophosphates are organic anions[103,104].

Uptake studies performed on MRP4-enriched isolated membrane vesicles and efflux experiments on *MRP4*-transfected cells have made it possible to identify various physiological substrates for the transporter[88]. Among the first substrates identified are cAMP and cGMP (Table 1)[101]; however, cAMP and cGMP levels remain relatively unaffected on a whole cell level[92,106]. Still, evidence suggests that cyclic nucleotide signaling is highly compartmentalized and that MRP4 was responsible for their regulation at a microdomain level and not at a whole cell level[95]. The correlation of MRP4 with cyclic nucleotides was studied in the gut epithelium, wherein a functional and physical coupling of MRP4 with a CFTR chloride channel was observed via a scaffolding protein. This resulted in an efflux of cAMP via the MRP4 transporter[95]. This finding was interesting because *Mrp4*$^{-/-}$ mice were more susceptible to CFTR-mediated diarrhea[95]. In addition, because high levels of MRP4 were found in the apical membrane of the proximal tubules in the nephron, MRP4 could possibly play a critical role of regulating the cAMP and cGMP levels in urine, in turn affecting the levels of water and salt homeostasis. However, there are no direct data to support this hypothesis *in vitro*[106,107]. Furthermore, *in vivo* data are also not promising, with up- and down-regulation of MRP4 in rats showing no correlation with the excretion rates of nucleotides[108,109]. Another study involving MRP4 inside-out membrane vesicles showed that MRP4 is responsible for efflux of second messenger cGMP from erythrocytes[101,110-114]. However, there was no *in vivo* study to support these findings, hence the physiological relevance of cGMP efflux by MRP4 from erythrocytes is questionable[104,110,111]. Nevertheless, the MRP4 membrane vesicle study showed that MRP4 could limit base/nucleoside analog accumulation in erythrocytes, which could affect the ability of the erythrocytes to function as carriers of xenobiotics like 6-TG and 6-MP[115].

Through the same MRP4 inside-out vesicle-mediated uptake studies, bile salts and urates (products of human purine) are identified to be physiological substrates of MRP4[98,116,117]. However, very low levels of bile salt and urate transport are observed with MRP4, making generation of *Mrp4* knockout mice necessary for further analysis of their transport. It was reported that fernesol X-activated receptor (*Fxr*)−knockout mice, which had lower levels of the major canalicular bile salt export pump (BSEP/ABCB11), had increased *Mrp4* mRNA levels[118]. Later on, similar results were reported in rats where relatively low levels of Mrp4 protein in the liver was increased significantly under hepatic stress[109,119,120]. In addition to its role in the liver, MRP4 also significantly impacts various other tissues where it is expressed, such as vascular smooth muscle, intestine, and blood cells[119]. MRP4 is also known to transport leukotrienes and prostanoids[119]. Apart from the up-regulation of MRP4 through long-term exposure to nucleoside-based drugs, MRP4 is also reported to be up-regulated in patients with neuroblastoma. However, no conclusive evidence has been provided to link drug resistance with MRP4 activity to date[120-122].

MRP5/ABCC5

MRP5 is encoded by a gene located on the 3q27 chromosome and contains 1437 amino acids[123]. Similar to MRP4, the identification of MRP5 was mainly based on expression sequence tag data analysis followed by cDNA fragment cloning[55,90]. MRP5 is widely expressed, with the highest levels occurring in the heart, brain, lungs, and skeletal muscles[55,124,125]. Structurally, MRP5 resembles MRP4 in that it lacks MSD_0. The two proteins differ at the NH_2 terminus, where MRP5 has 95 extra amino acids compare to 1325 amino acid of MRP4[123-125]. The function of this additional segment has still not been elucidated[123]. Membrane localization of MRP5 in *MRP5*-transfected polarized MDCKII cells was found to be in the basolateral membrane[126]. However, the transporter was located intracellularly, with only minor expression in the plasma membrane, in HEK293 cells[126]. Similar to MRP4, MRP5 can also transport cGMP and thus reduce its intracellular availability. Because of its widespread expression, MRP5 may affect the nitric oxide/cGMP pathway, which could ultimately lead to irregularities in muscle contractions[127,128]. Indeed, expression of MRP5 in the heart affected the muscle tone and contractility of cardiomyocytes[129,130]. Due to the abundant presence of Mrp5 in the brain pyramidal neurones and astrocytes, which are the centers for cell signaling in the brain, it reduces the intracellular cGMP levels, which results in inhibition of the Na^+/H^+ exchanger in rat astrocytes, leading to a decrease intracellular pH value[131-133]. MRP5 expression in the capillary endothelial cells of various tissues such as the heart and brain has protective and barrier functions[129,134]. In addition, MRP5 expression was reported in the basal membrane of syncytiotrophoblasts and around the fetus. Given that fact of its presence in the monocytes obtained from the peripheral whole blood cells in leukemic patients, MRP5 may play a role in the development of resistance to cancer chemotherapy[135].

PMEA, one of the important components of the antiretroviral therapy, is an acyclic nucleoside prototype, which is a potent inhibitor of HIV reverse transcriptase. To investigate the mechanism of resistance to antiviral drugs, Robbins *et al.*[136] developed a PMEA-resistant cell line and found that resistance to PMEA was mainly due to an increase in drug efflux, suggesting with a high possibility that a transporter was involved in this process.

Schuetz *et al.*[89] followed up this work and found ABC transporters, mainly MRP4, to be responsible for the efflux. Because MRP4 and MRP5 have structural similarities, Wijnholds *et al.*[126] investigated if they share similar substrates, and through an assay involving a radiolabeled hydrophobic PMEA precursor, they discovered that MRP5 serves as an active transporter of the drug. Subsequently, the same group demonstrated via similar studies that MRP5 confers resistance to 6-MP and 6-TG and their analogs[126], but these results were not reproducible[103]. 5-Flourouracil (5-FU) is an antimetabolite that very closely resembles nucleosides, making it possible that 5-FU could be a substrate of MRP5. This hypothesis was later confirmed[137], making 5-FU the only antimetabolite to be transported by MRP5. However, these results must be further validated to reduce controversy[126].

Physiologically, MRP5 is known to transport cAMP and cGMP, as was first demonstrated by Jedlitschky *et al.*[138] who used inside-out membrane vesicles obtained from human erythrocytes to show that MRP5 had a higher affinity for cGMP than that for cAMP (Table 1)[138]. cGMP transport via MRP5 was blocked by inhibitors such as probenecid, zaprinast, trequinsin, and sildenafil, indicating that the transport was due to an amphiphilic anionic transporter[89,111,139]. This is important because cGMP is the main mediator of nitric oxide (NO) and natriuretic peptides–mediated signaling, which control muscle relaxation, neutrophil degranulation, and platelet aggregation[140]. When cGMP competes with other substrates of MRP5 for transport, the half-maximal concentration of cGMP was 1 mmol/L, which was high for cellular levels[103]. Hence, researchers believe MRP5 to be an overflow pump with a very low affinity for cGMP, decreasing the levels of cGMP when it is overly synthesized[106]. Inhibitors for the pump have been previously reported by Jedlitschky *et al.*[138], but the results seemed controversial because other laboratories failed to replicate these results.

MRP6/ABCC6

MRP6 consists of three MSDs with five, six, and six TMDs, respectively, as well as two conserved NBDs. Due to its structural similarities with MRP1, it has been classified among the C subfamily of the ABC transporters. MRP1 and MRP6 both share almost 41% structural similarity[141,142]. *Mrp6* was first cloned in rat liver[69], and later it was cloned in humans and mice[143-145]. Mutations within the *MRP6* gene have been associated with genetic abnormalities of the autosomally inherited connective tissue disorder called pseudoxanthoma elasticum (PXE). To date, 90 distinct disease-causing mutations have been identified and reported in 31 exons of *MRP6*[146-152].

PXE has been characterized by dystrophic elastic fibers in the skin, retina, and large blood vessels, leading to baggy skin, loss of vision, and calcification of blood vessels[146,153]. The association of MRP6 with this disorder was unexpected because MRP6 was found to be localized mainly in the liver and kidney and with low and even undetectable levels in other tissues[141,143,154,155]. The localization of MRP6 was previously quite controversial, as rat Mrp6 was localized on the basolateral and canalicular plasma membrane of hepatocytes[144] whereas human MRP6 was present only on the basolateral membrane of hepatocytes[156]. Human and mouse kidney proximal tubules in *MRP6*-transfected MDCKII epithelial cells showed a basolateral manifestation of MRP6[145,157]. These findings provided new insight into the role of MRP6 in PXE, indicating that the disease could be a result of the absence of a substance that is normally excreted from the liver or kidney in the blood and is involved in tissue homeostasis[158]. Human *MRP6* transcripts were later detected in the skin, blood vessels, and retina with the help of reverse transcription–PCR. This was also confirmed via mouse immunohistochemical experiments wherein *Mrp6* transcripts were found to be expressed in the skin, retina, and aorta[145,146].

Substrate analysis studies of MRP6 showed that it is a lipophilic anionic pump, and that the substrates of MRP6 include drugs such as cyclopentapeptide BQ123[144]. Studies with *MRP6*-transfected Chinese hamster ovary (CHO) cell lines indicated that MRP6 functions as a drug efflux pump[159] and that MRP6 is capable of conferring very low levels of cellular resistance to etoposide and teniposide. In addition, studies suggest that MRP6 confers low levels of resistance to anthracyclines and cisplatin[159].

MRP6 can transport GSH conjugates such as LTC4 and n-ethylmaleimide-glutathione; however, it failed to pump out any glucuronate conjugates such as $E_217\beta G$[159,160]. These results indicate that MRP6 is a lipophilic anionic transporter and also that mutations involved in PXE may lead to a loss in activity in transporting these substrates.

MRP7/ABCC10

Similar to MRP1, MRP2, MRP3, and MRP6, MRP7 has three MSDs and two NBDs (Figure 1). Hopper *et al.*[161] used reverse transcription–PCR to analyze *MRP7* transcript expression and reported a low level in the skin, testis, spleen, stomach, colon, kidney, heart, and brain[161]. However, another group discovered that *MRP7* transcript expression was highest in the pancreas, followed by the liver, placenta, lungs, kidneys, brain, ovaries, lymph nodes, spleen, heart, leukocytes, and colon[162]. Kao *et al.*[163] discovered a splice variant of MRP7 that is truncated at its NH_2 terminus and has a 15–amino acid deletion between MSD2 and NBD2. MRP7 is a lipophilic

anion transporter whose physiological functions are so far unknown. However, one group found a potential role in the suppression of natural killer (NK)–mediated lysis[164]. To date, factors regulating MRP7 expression are unknown. However, MRP7 induction was found in doxorubicin-treated MCF7 cells[162].

Following the discovery of MRP7, Hopper-Borge *et al.*[97] analyzed the drug resistance profile of MRP7 using *MRP7*-transfected HEK293 cells. Similar to other MRPs, MRP7 can also confer resistance to several natural product anticancer drugs. A high level of resistance was observed against docetaxel, a microtubule stabilizing agent, whereas a moderate level of resistance was observed against paclitaxel[97]. In addition, MRP7 also confers resistance to microtubule destabilizing vinca alkaloids such as vincristine and vinblastine[97]. Recently, it was discovered that MRP7 might also be associated with vinorelbine resistance in NSCLC[165,166]. Resistance to taxanes by MRP7 is unique, as no other MRP member confers resistance to paclitaxel[166]. In addition, Hopper-Borge *et al.*[167] found that MRP7 can also confer resistance to nucleoside-based agents such as the anticancer drugs (Ara-C and gemcitabine) and the antiviral drugs (2',3'-dideoxycytidine and PMEA)[167]. Another microtubule-stabilizing agent, epothilone B, was also identified as a substrate for MRP7 (Table 2) in the same study[167]. In a separate study, ectopic expression of Mrp7 was observed in mouse embryo fibroblasts deficient in P-gp and Mrp1[167]. The same group also reported that MRP7 has a broad resistance profile for natural product agents, conferring high levels of resistance to docetaxel (46-fold), paclitaxel (116-fold), SN-38 (65-fold), daunorubicin (7.5-fold), etoposide (11-fold), and vincristine (56-fold)[167]. In addition, buthionine sulfoximine did not have any effect on MRP7-mediated resistance to docetaxel or Ara-C, suggesting that MRP7 transport does not involve GSH[167]. In an *in vitro* study, mouse fibroblast cells from *Mrp7* knockout mice were sensitive to several natural anticancer drugs such as docetaxel, paclitaxel, vincristine, and Ara-C, confirming the previously characterized resistance profile of MRP7[97]. These cells also showed increased levels of drug accumulation relative to wild-type controls. In the same study, *Mrp7* knockout mice exhibited higher lethality associated neutropenia and marked bone marrow toxicity upon treatment with paclitaxel, indicating that Mrp7 is indispensable for health and viability. Taken together, these results show that MRP7 is an endogenous resistance factor for taxanes, vinca alkaloid anticancer drugs, and nucleoside analogs[168]. In contrast, taccalonolides, another class of natural product microtubule stabilizers, do not succumb to P-gp– or MRP7-mediated resistance[169]. Studies investigating MRP7 expression in lung, breast, and ovarian tumor specimens would be interesting because this protein confers resistance to paclitaxel and vincristine, which are the mainstays of treatment for these particular cancers.

In a transport study involving membrane vesicles from *MRP7*-transfected HEK293 cells, $E_2 17\beta G$, a prototypical substrate of many MRPs, was identified as a substrate of *MRP7*, with a K_m value of 57.8 μmol/L. As $E_2 17\beta G$ is a glucuronide conjugate, MRP7 might be involved in phase III detoxification. Chen *et al.*[170] found that MRP7 had modest activity in transporting LTC4 but did not transport glycocholic acid, taurocholic acid, MTX, folic acid, cAMP, or cGMP, which are substrates of other MRP family members (Table 1). They also determined that the biochemical features of MRP7 matched the core features of other MRPs capable of transporting lipophilic anions, though MRP7 had limited substrate selectivity[170]. Furthermore, they observed that the transport of $E_2 17\beta G$ was competitively inhibited by amphiphiles, such as LTC4, glycolithocholate 3-sulfate, and MK571, as well as lipophilic agents, such as cyclosporine A[170]. This supports the notion that the MRP7 substrate binding pocket has sites for anionic and lipophilic moieties.

MRP7 expression has been reported in salivary gland adenocarcinoma and NSCLC[165,171,172]. In addition, *MRP7* transcripts have been detected in the HepG2 liver cancer cell line and two prostate cancer cell lines (CWR22Rv1 and TSU-PR1)[173], as well as in breast, lung, colon, prostate, ovarian, and pancreatic tumor specimens[162]. However, information about MRP7 expression in tumors is still limited.

MRP8/ABCC11

MRP8 is a newly found member of the MRP family. The *MRP8* gene contains 29 exons and encodes a protein predicted to contain 1382 amino acids[174,175] that is structurally similar to MRP4 and MRP5, with 2 MSDs, 2 NBDs, and 12 TMDs. Sequence comparisons done between MRP8 and the other family members indicate its close resemblance with MRP5[174,176]. Bera *et al.*[174] was the first to discover *MRP8* with the help of a gene prediction program and expressed sequence tag (EST) database mining. MRP8 is widely expressed within the human body, with the highest levels occurring in the liver, brain, placenta, breasts, and testes (Table 1)[174,175]. This widespread expression pattern is purportedly due to MRP8 spliceoforms[175]. MRP8 is known to play a role in the human central and the peripheral nervous system such that the expression of MRP8 is associated with the efflux of dehydroepiandrosterone 3-sulfate (DHEAS), a neuromodulatory steroid[177].

The resistance profile of MRP8 was determined using pig kidney epithelial (LLC-PK1) cells, which ectopically expressed the pump. Studies done using inside-out membrane vesicles showed that MRP8 does

not share the same substrates as MRP5, despite their structural similarities. Instead, MRP8 has a similar substrate specificity to MRP4, as both transporters can pump DHEAS, estrone-3-sulfate (E_1S), folates, monoanionic bile acids, and GSH and glucuronate conjugates[96,100,101]. Nevertheless, there are some marked differences among the two transporters. MRP4 is strictly dependent upon GSH and can transport prostaglandins, PGE1, and PGE2[116,178]. In contrast, MRP8 transports monoanionic bile acids in the absence of GSH, and it is unable to transport PGE1 and PGE2[179]. MRP8 can confer resistance to PMEA, 2',3'-dideoxycytidine, 5-FU, MTX, and Ara-C (Table 2)[180]. Transport analysis using MRP8 membrane vesicles showed that MRP8 can transport a wide range of compounds, including nucleotide analogs; lipophilic anions such as natural and synthetic glutathione conjugates LTC4 and DNP-SG; $E_217\beta G$; monoanionic bile acids glycocholate and taurocholate; steroid sulfates such as DHEAS and E_1S; folic acid; and MTX (Table 1)[179]. Under basal and stimulated conditions, expression of MRP8 resulted in decreased intracellular concentrations and increased extracellular concentration of cAMP and cGMP[177].

The results from various studies describing the substrates and expression pattern of MRP8 suggest that it could play a major role in maintaining the normal body functions. With its ability to transport monoanionic bile acids and its expression in the liver, MRP8 may affect homeostasis within hepatocytes[179]. In addition, with the ability to transport glucuronidated and sulfated steroids like $E_217\beta G$, DHEAS, and E_1S, and with its expression in areas such as the breasts, testes, and prostate, MRP8 could play a crucial role in determining how these hormone-regulated tissues would respond to sex steroids[179]. Clinically, MRP8 is reported to be highly expressed in breast cancer patients[174]. In addition, overexpression of MRP8 is reportedly significantly related to lower overall survival in acute myelogenous leukemia (AML) patients, implicating it as a possible biomarker[181]; however, MRP8 expression in normal tissues still needs to be established. Indeed, more studies are required to establish a link between MRP8 expression and the possible clinical significance in MDR[181].

MRP9/ABCC12

MRP9 is the last member within the MRP family to be cloned. There will likely be no further cloning because the sequence is complete, and the last member of the family, ABCC13, is reported to be a pseudogene[182-184]. MRP9 is located in close proximity to MRP8 in a head-to-tail orientation at chromosomal region 16q12.1[184]. This locus is implicated as a potential candidate gene(s) for paroxysmal kinesigenic choreoathetosis (PKC), a disorder involving abnormal involuntary movements[184] and infantile convulsions with paroxysmal choreoathetosis (ICCA)[91,176]. The MRP9 sequence, which is similar to MRP8 sequence, shares 44% identity and 55% sequence similarity with MRP5 sequence[125,185]. MRP9 also contains 2 MSD and 12 TM helices; however, some researchers have suggested that it has only 1 NBD and 8 TM helices[185]. *MRP9* encodes a protein of 1359 amino acids that does not undergo N-glycosylation[185]. According to reports, there are two *MRP9* transcripts, one 4.5 kb in length that is expressed in breast cancer, normal breasts, and the testis, and the other 1.3 kb in length that is expressed in the brain, skeletal muscle, and ovaries[185]. However, a recent study showed only full-length Mrp9 in testicular germ cells and mouse sperm[186]. No report has been published about the substrate profile of MRP9[185]. Using membrane vesicles prepared from insect Sf9 cells, no transport was observed for cGMP, cAMP, MTX, GSH, glycocholic acid, taurocholic acid, DHEAS, or $E_217\beta G$[187].

Conclusions

MRPs have variable tissue distributions, cellular localizations, and pharmacological and physiological functions (Table 1). The uniqueness of MRPs is that they confer resistance to a range of anticancer drugs that is broader than the range of drugs handled by P-gp, the first and the most widely studied ABC transporter. MRP1 and MRP2 confer resistance to natural anticancer drugs such as vinca alkaloids and MTX (antifolate), which are hydrophobic. MRP3 confers resistance to MTX and epipodophylotoxins. The most intriguing feature of MRPs 1–3 is that they provide a transport facility for compounds (drugs, xenobiotics, or physiological substrates) conjugated with GSH, glucuronide or sulfate. MRP4 and MRP5 confer resistance to nucleobase and nucleoside analogs such as PMEA, 6-MP, and 6-TG. MRP8 confers resistance to PMEA but not to 6-MP or 6-TG. MRP7 confers resistance to almost every category of drugs, ranging from natural anticancer drugs to nucleoside analogs and epothilone B. Extensive studies performed on these transporters revealed that they are expressed in tumor tissues. This makes them a prime suspect in the development of MDR apart from P-gp and BCRP. However, there are still insufficient data from which to derive a definite conclusion about MRP expression and the development of clinical MDR. Further studies are required to confirm the role of individual MRP members and target them to confront MDR.

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Review

Breast cancer resistance protein (BCRP/ABCG2): its role in multidrug resistance and regulation of its gene expression

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Abstract

Breast cancer resistance protein (BCRP)/ATP-binding cassette subfamily G member 2 (ABCG2) is an ATP-binding cassette (ABC) transporter identified as a molecular cause of multidrug resistance (MDR) in diverse cancer cells. BCRP physiologically functions as a part of a self-defense mechanism for the organism; it enhances elimination of toxic xenobiotic substances and harmful agents in the gut and biliary tract, as well as through the blood-brain, placental, and possibly blood-testis barriers. BCRP recognizes and transports numerous anticancer drugs including conventional chemotherapeutic and targeted small therapeutic molecules relatively new in clinical use. Thus, BCRP expression in cancer cells directly causes MDR by active efflux of anticancer drugs. Because BCRP is also known to be a stem cell marker, its expression in cancer cells could be a manifestation of metabolic and signaling pathways that confer multiple mechanisms of drug resistance, self-renewal (stemness), and invasiveness (aggressiveness), and thereby impart a poor prognosis. Therefore, blocking BCRP-mediated active efflux may provide a therapeutic benefit for cancers. Delineating the precise molecular mechanisms for *BCRP* gene expression may lead to identification of a novel molecular target to modulate BCRP-mediated MDR. Current evidence suggests that *BCRP* gene transcription is regulated by a number of trans-acting elements including hypoxia inducible factor 1α, estrogen receptor, and peroxisome proliferator-activated receptor. Furthermore, alternative promoter usage, demethylation of the *BCRP* promoter, and histone modification are likely associated with drug-induced BCRP overexpression in cancer cells. Finally, PI3K/AKT signaling may play a critical role in modulating BCRP function under a variety of conditions. These biological events seem involved in a complicated manner. Untangling the events would be an essential first step to developing a method to modulate BCRP function to aid patients with cancer. This review will present a synopsis of the impact of BCRP-mediated MDR in cancer cells, and the molecular mechanisms of acquired MDR currently postulated in a variety of human cancers.

Key words BCRP, ABCG2, multidrug resistance (MDR), transporter, gene expression, tyrosine kinase inhibitors, cancer stem cells

Multidrug resistance (MDR) is a phenomenon in which cancer cells simultaneously become resistant to structurally unrelated chemotherapeutic agents when exposed to a single chemotherapeutic drug. The development of MDR in the course of chemotherapy has been considered as a major obstacle in cancer treatment. For the last three decades, the biological causes underlying MDR have been extensively studied and attributed to diverse molecular mechanisms. Active efflux mediated by drug efflux pumps has been described in a wide variety of cancer cells since *MDR1*, which encodes the membrane transport protein P-glycoprotein (P-gp), was isolated from KB cells selected with vinblastine in 1986[1]. P-gp was the first human ATP-binding cassette (ABC) transporter protein to be identified and is classified as the first member of the B

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