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Multidrug Resistance Proteins (MRPs/ABCCs) in Cancer Chemotherapy and Genetic Diseases

Zhe-Sheng Chen and Amit K. Tiwari

Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. John's University, Queens, NY 11439, USA.

Abstract

The ATP-binding cassette (ABC) transporters are a superfamily of membrane proteins that are best known for their ability to transport a wide variety of exogenous and endogenous substances across membranes against a concentration gradient via ATP hydrolysis. There are seven subfamilies of human ABC transporters, one of the largest being the 'C' subfamily (gene symbol *ABCC*). Nine *ABCC* subfamily members, the so-called Multidrug Resistance Proteins (MRPs) 1-9, have been implicated in mediating multidrug resistance in tumor cells to varying degrees as the efflux extrude chemotherapeutic compounds (or their metabolites) from malignant cells. Some of the MRPs are also known to either influence drug disposition in normal tissues or modulate the elimination of drugs (or their metabolites) via hepatobiliary or renal excretory pathways. In addition, the cellular efflux of physiologically important organic anions such as leukotriene C_4 and cAMP is mediated by one or more of the MRPs. Finally, mutations in several MRPs are associated with human genetic disorders. In this review article, the current biochemical and physiological knowledge of MRP1-MRP9 in cancer chemotherapy and human genetic disease is summarized. The mutations in *MRP2/ABCC2* leading to conjugated hyperbilirubinemia (**Dubin-Johnson syndrome**) and in *MRP6/ABCC6* leading to the connective tissue disorder **Pseudoxanthoma elasticum** are also discussed.

Keywords

ABCC Transporters; Dubin-Johnson Syndrome; Multidrug Resistance; Multiple Resistance Protein; Pseudoxanthoma Elasticum

Introduction

The 48 human ABC genes have been subdivided into seven subfamilies from ABC-A through -G based on their relative sequence similarities. Subfamily C contains thirteen members and nine of these drug transporters are often referred to as the Multidrug Resistance Proteins (MRPs) (Fig. 1, Table 1) [1]. The MRP proteins are found throughout the nature and they mediate many important functions. Of the nine MRP proteins, four of them, MRP4, 5, 8, 9 (*ABCC4*, 5, 11 and 12), have a typical ABC structure with four domains, comprising two membrane spanning domains (MSD1 and MSD2), each followed by a nucleotide binding domain (NBD1 and NBD2) (Fig. 2) and these are referred to as the "short" MRPs. The so-called "long" MRPs, MRP1, 2, 3, 6, 7 (*ABCC1*, 2, 3, 6 and 10), have an additional fifth domain, MSD0, at their NH_2 -terminus. MSD1 and MSD2 (which each contains 6 transmembrane α -helices (TMs)) form the translocation pathway through which

substrates cross the membrane, while the two NBD proteins associate in a head-to-tail orientation to form a “sandwich dimer” that comprises two composite nucleotide binding sites [2-6]. Certain functionally important sequence variations in the NBDs of the MRP-related proteins, which are usually highly conserved throughout the ABC superfamily, contributes to some of the differences in how members of this subfamily interact with ATP and the products of its hydrolysis [3, 7, 8].

A significant number of structurally diverse molecules can be transported across membranes by the MRPs [3-5, 9, 10]. Although some of the MRPs share a limited degree of overlap in their substrate specificity (at least *in vitro*), there are usually substantial differences in the transport kinetics observed for a common substrate. Differences in the tissue distribution pattern of the MRPs, as well as their membrane localization (apical vs basolateral) in polarized epithelial and endothelial cells, are also important determinants of their distinct pharmacological and physiological functions (Fig. 2).

MRP1/ABCC1

MRP1 (gene symbol *ABCC1*) was the first of the drug transporting ABCC proteins to be cloned and was found to be highly overexpressed in a doxorubicin-selected multidrug resistant human lung carcinoma cell line H69AR [2]. MRP1 plays an important role in drug and xenobiotic disposition in normal cells and helps protect certain tissues from cytotoxic insults [9]. Indeed, the tissue distribution of MRP1 is consistent with its role in limiting the penetration of certain cytotoxic agents through a number of blood-organ interfaces. In this way, MRP1 contributes to so-called “pharmacological sanctuary sites” in the body, such as the blood-brain barrier and the blood-testis barrier [9]. The most direct evidence demonstrating a role for MRP1 in tissue defense has come from studies of mice in which the *Mrp1/Abcc1* gene has been disrupted. Although these knock-out mice are viable and fertile, they exhibit increased chemosensitivity in certain tissues such as the seminiferous tubules, the intestine, the oropharyngeal mucosa and the choroid plexus [11-13].

Clues to the physiological function of MRP1 were first provided by *in vitro* data indicating that it was a high affinity transporter of glutathione (GSH, γ -Glu-Cys-Gly) conjugated arachidonic acid derivative leukotriene C₄ (LTC₄) [14]. The LTC₄ is a pro-inflammatory arachidonic acid derivative that is involved in asthmatic and allergic reactions as well as smooth muscle constriction and vasoconstriction [15].

Confirmatory *in vivo* evidence that LTC₄ export is a physiological function of MRP1 was provided by the observation that *Mrp1*^{-/-} mice exhibited diminished inflammatory responses associated with defective LTC₄ efflux [11]. In addition to LTC₄, there are other transported conjugates that are endogenous metabolites like LTC₄, while many others are the products of the GSH-S-transferase (GST) catalyzed conjugation of xenobiotics with GSH [4,10]. Therefore, MRP1 is sometimes referred to as a GS-X efflux pump - a term that also applies to MRP2 (see below) [16]. However, MRP1 is a much more versatile transporter than this term implies since it also transports organic anions conjugated to glucuronate as well as sulphate. Again, some of these conjugates are endogenous metabolites (e.g. estradiol glucuronide, estrone sulphate), whereas others are conjugates of xenotoxins, such as the glucuronide conjugate of the tobacco-specific carcinogen, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanol (NNAL) [17]. One notable but often overlooked feature of MRP1 is the marked differences between the substrate specificity of MRP1 from primates and *Mrp1* from other species, even though the amino acid sequence similarity is >90% for all species [3,18]. For example, MRP1/*Mrp1* from humans and macaque monkeys can transport estradiol glucuronide and anthracycline antibiotics, such as doxorubicin, whereas this function is lost in *Mrp1* from rat, mouse, dog, or cow.

MRP1 has a number of complex interactions with GSH [10]. This cellular tripeptide antioxidant is most noted for its crucial role in protecting cells from the deleterious effects of oxidative stress. It is required in xenobiotic metabolism to form GS-X conjugates, which are then exported by MRP1 (or MRP2). The observation that GSH levels in some tissues of *Mrp1*^{-/-} mice are elevated up to 2-fold has been interpreted as *in vivo* evidence that GSH is also an MRP1 substrate [13]. *In vitro* GSH is transported by MRP1 (and MRP2) with low affinity, whereas the pro-oxidant glutathione disulphide (GSSG) is a relatively higher affinity substrate of these transporters [19]. Consistent with the ability to transport these critical sulfhydryls (SH), accumulating evidence indicates that MRP1 plays an important modulatory role in cellular oxidative stress and redox homeostasis [20]. MRP1-mediated GSH transport can be stimulated by a range of xenobiotics, and in the case of bioflavonoids like apigenin, it appears that these stimulatory compounds are not MRP1 substrates [21, 22]. The *Vinca* alkaloid vincristine, however, markedly enhances GSH transport by MRP1, whereas GSH stimulates vincristine transport [23, 24]. Thus, in this instance, GSH appears to be co-transported with (or cross-stimulates transport of) the drug. Finally, while many conjugated organic anions are transported by MRP1, there are some instances where this transport is dependent on or enhanced by GSH. For example, the transport of the conjugates NNAL-*O*-glucuronide and estrone sulphate is enhanced by GSH; however, in contrast to vincristine transport, GSH only stimulates the process [17, 25]. The biological activities of GSH are generally attributed to the proton-donating properties of the cysteine SH moiety. However, this is not the case for its stimulatory effects on MRP1 activity. For example, drug transport by MRP1 can also be stimulated in non-reducing tripeptides, where the Cys residue has been replaced by another amino acid such as Leu or is modified with a short chain alkyl moiety (e.g. *S*-methyl GSH) [23, 26].

The amino acids important for the substrate specificity of MRP1 are frequently located in the TMs, particularly those in MSD1 and MSD2 which form the substrate translocation pathway through the membrane. Many of these functionally important amino acids have been identified by site-directed mutagenesis studies [4, 27, 28]. These and other studies are providing important insights into the molecular features of MRP1 that govern the proper assembly and expression of this transporter at the plasma membrane.

In tumor cells, the 190 kDa MRP1 can confer resistance to not only doxorubicin, but many other widely used antineoplastic drugs, including methotrexate (MTX), daunorubicin, vincristine and etoposide [5, 23, 29, 30]. Some of the newer so-called 'targeted' agents (e.g. certain tyrosine kinase inhibitors (TKIs)) that modify various signal transduction pathways, can also be transported by MRP1 [31]. MRP1 has been reported to be highly expressed in leukemias, esophageal carcinomas, and non-small cell lung cancer [32]. In addition, several reports have correlated MRP1 expression with clinical outcome [33]. Although, MRP1 mRNA and/or protein have been frequently detected in patient tumor samples, the overall contribution of this drug transporter to clinical drug resistance is still not well defined [3].

MRP2/ABCC2

Mrp2 (gene symbol *ABCC2*) was first identified as a hepatocellular canalicular multiple organic anion transporter (cMOAT) and was originally cloned from rat liver using strategies that took advantage of its structural similarity to human MRP1 [34, 35]. Subsequently, the human MRP2, rabbit, mouse, and canine Mrp2 cDNAs were cloned and the five orthologs showed a high degree of amino acid similarity (77–83%) [36–39]. MRP2 and MRP1 share a 49% amino acid identity. In contrast to MRP1, MRP2 has a markedly different expression pattern and is primarily expressed in the apical plasma membrane of hepatocytes, the brush-border membrane of kidney proximal tubules, and the intestine [40–42]. In addition, MRP2

mRNA has also been detected in peripheral nerves, gallbladder, placental trophoblasts and CD4+ lymphocytes [43-45].

Similar to MRP1, MRP2 can transport many different substrates [39, 46]. However, although MRP2 can transport certain hydrophobic compounds in the presence of GSH [47], MRP2 also can transport organic anions including sulfate, glucuronide, and GSH conjugates [39, 46, 48]. In addition, MRP2 is also responsible for the biliary elimination of certain endogenous conjugates, such as LTC₄ and conjugated bilirubins (Table 1) [39, 48].

The physiological role of MRP2 was confirmed using *Mrp2*^{-/-} mice [49], which are healthy and have no obvious phenotypic abnormalities. MRP2 appears to play a role in eliminating endogenous metabolites, as well as xenobiotics and their metabolites. In the mouse liver, *Mrp2* mainly transports bilirubin glucuronides into the bile. However, there is an increase in the levels of bilirubin and glucuronides in the serum and urine of these mice. Moreover, these mice show decreased biliary excretion of bilirubin glucuronides and total GSH, and decreased biliary excretion of the *Mrp2* substrate dibromosulfophthalein [49]. In the rat, where hepatic expression of *Mrp2* is relatively high, *Mrp2* may play a greater role in biliary excretion of conjugated organic anions compared with other species [50, 51]. Indeed, in *Mrp2*-deficient rats, glucuronide conjugates of acetaminophen, hydroxyphenobarbital, phenolphthalein, and 4-methylumbelliferone were not excreted into bile or the urine [52-55]. However, when the clearance of acetaminophen sulphate, acetaminophen glucuronide, 4-methylumbelliferyl sulphate, 4-methylumbelliferyl glucuronide, harmol sulphate and glucuronide was examined in *Mrp2*-deficient mice, only the clearance of 4-methylumbelliferyl glucuronide was decreased. Since MRP2 is known to mediate elimination of drug conjugates, there has been considerable interest in understanding its role in drug-drug interactions. Currently, a broad range of structurally unrelated compounds have been shown to inhibit MRP2 activity. Examples include probenecid, a drug used to treat gout, and MK-571, a leukotriene receptor antagonist [56, 57]. In addition, montelukast, a drug used in the pharmacotherapy of asthma, has been shown to inhibit the efflux of paclitaxel and saquinavir from MRP2-overexpressing cells [58]. Additional pharmaceutical modulators of MRP2 include glibenclamide, rifampicin, indomethacin, cyclosporin A, and the antibiotics such as azithromycin, fusidate and gatifloxacin [57, 59-61].

Similar to MRP1, MRP2 has two MSDs characteristic of ABC transporters, in addition to a third NH₂-terminal MSD (MSD0) and a COOH-terminal region. Sequences within MSD0 of MRP2 are required for its activity and plasma membrane trafficking, as are sequences within its COOH-terminal region [62]. In addition, this latter region of MRP2 contains a putative PDZ binding motif that is involved in membrane targeting via its interaction with scaffolding proteins such as radixin in hepatocytes, as the localization of *Mrp2* to the hepatocanicular membrane is impaired in radixin knockout mice [63, 64]. Basic residues in the TM helices of MRP2 are important for recognition and translocation of its substrates [65], as they are for MRP1 [66]. For example, basic residues in TM6, TM9, TM16, and TM17 are involved in the binding of glutathione conjugates and an amino acid in TM11 promotes the stable expression of MRP2 [65]. In addition, an aromatic amino acid, Trp1254 in TM17, plays a crucial role in the ability of human MRP2 to transport methotrexate [67]. It is thought that MRP2 contains at least two distinct binding sites: one site for drug transport and a second site that allosterically regulates the former due to the complex inhibition and stimulation patterns obtained from the vesicle transport assay in the presence of different modulators [68].

MRP2 is expressed in unselected lung, gastric, renal and colorectal tumor cell lines [69]. Increased MRP2 mRNA levels have been reported in some cisplatin- and doxorubicin-resistant cell lines [36, 43]. MRP2 is also expressed in some solid tumors originating from

the kidney, colon, breast, lung, ovary, as well as in cells from patients with acute myelogenous leukemia [70, 71]. *In vitro* studies have reported that MRP2 transports a variety of anticancer drugs, including MTX, cisplatin, irinotecan, paclitaxel and vincristine (Table 1) [72-74]. Recently, Korita et al. reported that MRP2 expression determines the efficacy of cisplatin-based chemotherapy in patients with hepatocellular carcinoma [75].

MRP2 and Dubin-Johnson Syndrome

The Dubin-Johnson Syndrome (DJS) is an inherited autosomal recessive disorder characterized by chronic conjugated hyperbilirubinemia, impaired secretion of anionic conjugates from hepatocytes into the bile, and deposition of brown pigments in the liver [40, 76, 77]. A substantial number of mutations in human *MRP2* that result in deficiencies of MRP2 function have been identified since the late 1990's (Fig. 3B). These mutations (nonsense, missense, deletion, splice site) involve amino acids located throughout the protein and result in the absence of a functionally active MRP2 protein in the canalicular membrane [78-81]. The *Mrp2* deficient Eisai hyperbilirubinuria rats (EHBRs) and Groninger Yellow transporter rat strains are well established animal models of the human DJS [82-84].

MRP3/ABCC3

MRP3 (gene symbol *ABCC3*) was first cloned shortly after MRP2 [85-88], and within the MRP subfamily of the ABC transporters, it shares the closest degree of similarity (58% amino acid identity) with MRP1. Similar to MRP1, MRP3 is also expressed on the basolateral membranes of polarized cells [87, 88]. However, MRP3 (like MRP2) has a relatively more restricted tissue distribution pattern than MRP1. In humans, MRP3 is mainly expressed in the adrenal glands, kidney, small intestine, colon, pancreas, and gallbladder, with a lower magnitude of expression in the lungs, spleen, stomach, and tonsils [43, 85-88, 90, 91].

In the normal human liver, MRP3 expression is low and mainly limited to the basolateral membranes of bile duct epithelial cells and hepatocytes surrounding the portal tracts [88, 89]. However, MRP3/*Mrp3* expression is up-regulated in the cholestatic human [91] and rat liver [93] and in patients with the DJS who lack functional MRP2 in liver canalicular membranes. It appears that MRP3 plays a compensatory role for the loss of MRP2 in the liver [88]. In the normal rat liver, *Mrp3* mRNA expression is very low, but in *Mrp2*-deficient EHBR and TR-mutant rats, as well as in normal rats with ligated bile ducts, *Mrp3* expression is significantly increased [84, 92, 93].

The physiological function of MRP3 has been studied using indirect approaches, such as determination of tissue localization, upregulation in pathological conditions and substrate specificity, using *in vitro* assays. The *Mrp2*^{-/-}/*Mrp3*^{-/-} mouse model has provided a model for the direct investigation of the role of *Mrp3* in the disposition of endogenous compounds and xenobiotics. Only two studies on the physiological function of MRP3 using *Mrp3*^{-/-} mice have been published. The findings of these studies indicated that *Mrp3* does not play a significant role in the transport of major bile salts, but is probably involved in the hepatic sinusoidal excretion of glucuronidated compounds [94, 95]. Thus, the induction of cholestasis in *Mrp3*^{-/-} mice by bile duct ligation results in decreased concentrations of serum bilirubin glucuronide and increased hepatic concentrations of bile acid conjugates [94]. In humans, MRP3 may protect the liver from cholestasis that may occur from the toxic accumulation of hepato-toxic bile salts.

The pharmacological role of *Mrp3* as a basolateral excretory system has also been explored *in vivo* using *Mrp3*^{-/-} mice. Compared to wild-type mice, the levels of morphine-3-

glucuronide (M3G) in *Mrp3*^{-/-} mice are significantly increased in the liver and bile, whereas decreased in the plasma [94]. In addition, the antinociceptive potency of the M6G is also decreased in *Mrp3*^{-/-} mice compared to wild-type mice [96]. Similarly, in *Mrp3*^{-/-} mice in contrast to wild-type mice acetaminophen-glucuronide levels increased in the liver, whereas the plasma levels were significantly decreased [95]. *Mrp3*^{-/-} mice also exhibited normal unconjugated bile salt transport but decreased serum concentrations of glucuronide conjugates of bile acids [97].

MRP3/Mrp3 transports variety of amphipathic anions including glucuronate conjugates and monoanionic bile acids (Table 1) [88, 93, 98-100]. However, MRP3 has a very low affinity and capacity for the transport of GSH conjugates, and unlike MRP1 and MRP2, does not efflux GSH or require this tripeptide for drug transport [93, 98, 99]. Recently, Lagas et al. observed that the hepatobiliary excretion of etoposide was almost completely dependent on Mrp2, although *Mrp2*^{-/-} mice did not display elevated concentrations of etoposide in the liver, presumably because Mrp3 was able to transport etoposide to the blood circulation from the liver [101]. Lagas et al. also reported that *Mrp2*^{-/-}/*Mrp3*^{-/-} mice significantly accumulated etoposide glucuronide in the liver, whereas both single knockout animals did not, indicating that Mrp2 and Mrp3 provide alternative pathways for hepatic elimination of etoposide glucuronide [101]. Therefore, it appears that *Mrp3*^{-/-} mice have defects in the sinusoidal excretion of glucuronide conjugates formed in the liver, suggesting that MRP3/Mrp3 plays a crucial role in the basolateral elimination of bile acids and certain glucuronide conjugates *in vivo*.

An elevation in the levels of MRP3 expression has been detected in human hepatocellular carcinomas [102], primary ovarian cancer [103], and adult acute lymphoblastic leukemia (ALL) [104]. In addition, over-expression of MRP3 was predicted to be a prognostic factor in childhood and adult ALL and adult acute myeloid leukemia (AML) [104, 105]. MRP3 transports fewer anticancer substrates compared to MRP1 and MRP2, such as etoposide, teniposide and MTX (Table 1) [87, 99, 101]. In some instances, the expression of MRP3 is associated with clinically relevant resistance and poor chemotherapeutic response, however, further investigations are needed to confirm the role of MRP3 in conferring clinical MDR.

MRP4/ABCC4

MRP4 (gene symbol *ABCC4*) was first functionally identified as a transporter of 9-(2-phosphorylmethoxyethyl) adenine (PMEA), a nucleoside monophosphate antiviral agent [106]. MRP4 shares a 41% amino acid identity with MRP1. MRP4 is present at low levels in all normal tissues, with substantially higher levels found in the prostate [107]. MRP4 has intriguing membrane trafficking properties, in that it can localize to both basolateral and apical membranes in polarized cells, depending on the tissue where it is found. For example, in prostate tubuloacinar cells and hepatocytes, MRP4 localizes to the basolateral membrane, whereas in renal proximal tubules and the luminal side of brain capillaries, it is found at the apical membrane [98, 107, 108]. The mechanisms underlying the tissue specific membrane localization of MRP4 appear to involve interactions with different adaptor and scaffolding proteins in the different cell types, although the precise details of these interactions have only been partially elucidated [109, 110].

However, subsequent *in vitro* and in *Mrp4*^{-/-} mice studies suggested that this transporter has broad substrate specificity [98, 106, 111-113]. *Mrp4*^{-/-} mice have proven to be extremely valuable models in demonstrating the importance of Mrp4 in drug disposition and elimination. For example, topotecan accumulation in both brain tissue and in cerebral spinal fluid is enhanced in these animals [114]. This reflects the dual localization of Mrp4 at the basolateral membrane of the choroid plexus epithelium, and at the apical membrane of the

endothelial cells of the brain capillaries. Renal elimination of many drugs is also reduced in *Mrp4*^{-/-} mice [107, 115-117]. Furthermore, MRP4 inhibitors such as non-steroidal anti-inflammatory drugs (NSAIDs, e.g. celecoxib) may contribute to clinically significant kidney toxicity when cytotoxic agents are co-administered with NSAIDs [107, 118, 119]. *Mrp4*^{-/-} mice are also more sensitive to the hematopoietic toxicity of thiopurines [120]. In humans, a non-synonymous polymorphism in *MRP4* results in the production of an inactive transporter and it has been suggested that the increased sensitivity of some Japanese patients to thiopurine-based therapy may reflect the greater frequency of this polymorphism in the Japanese population [120].

In addition to its drug (and drug metabolite) transporting function, MRP4 mediates the cellular efflux of several endogenous metabolites that play critical roles in signaling pathways involved in processes such as differentiation, pain perception and inflammation. For example, MRP4 appears to be responsible for cellular efflux of cAMP (and cGMP) the second messengers [121]. The affinity of MRP4 for cAMP (and cGMP) is relatively low, raising questions about relevance in regulating intracellular levels of these cyclic nucleotides [98]. However, there is an increasing amount of evidence indicating that cyclic nucleotide signaling is highly compartmentalized, suggesting that MRP4 may be more involved in regulating local microdomain levels rather than whole cell concentrations of cAMP [122]. Several eicosanoids are substrates of MRP4, including prostaglandin E₂ (PGE₂), which is a known mediator of pain and inflammation and also have implications in tumor development, growth, angiogenesis, and response to cytotoxic chemotherapy, (Table 1) [86, 119, 123, 124]. Other reported substrates of MRP4 includes GSH, sulfated bile acids, GSH conjugated leukotriene B₄ (LTB₄) and LTC₄ (Table 1) [111, 125].

MRP4 has been implicated in the high proliferative growth of some tumors including prostate tumors and neuroblastoma [108, 112, 113]. Recently, the absence of MRP4 protein has been associated with a selective defect in ADP storage in platelet δ -granules, which in turn is associated with prolonged bleeding times and bleeding diathesis [126]. In addition to PMEA and other antiviral agents, MRP4 also confers resistance to anticancer agents including thiopurine analogs, MTX and topotecan (Table 1) [107, 111, 114, 127, 128]. Thiopurine analogs and most nucleoside-based chemotherapeutic drugs require intracellular phosphorylation before they can exert their pharmacologic activity. Consequently, MRP4 confers resistance to these agents by effluxing their anionic phosphate metabolites rather than the parent compounds [118, 128].

MRP5/ABCC5

MRP5 (gene symbol *ABCC5*) was identified as a result of database screening of sequence tags [43], but was first functionally characterized to transport nucleotide analogs by Wijnholds et al. [129]. MRP5 shares only a 38% identity with MRP1. MRP5 mRNA is expressed in most normal tissues at low levels, with maximum expression in skeletal muscle, heart brain and the cornea [43, 130-133]. Like MRP1, MRP5 is located on the luminal side of brain capillary endothelial cells, pyramidal neurons and subcortical white matter astrocytes [108, 134]. In polarized cells, MRP5 is preferentially localized in the basolateral membrane [108]. Interestingly, as gestational age increases, the levels of MRP5 mRNA decrease significantly [135].

Studies utilizing knockout mice have thus far yielded little insight into the functional activity of MRP5. *Mrp5*^{-/-} mice appear normal and are fertile with no known physiologically abnormal phenotype, at least up to 1 year [129]. Evidence suggests that MRP5 is also involved in the cellular extrusion of the second messengers, cGMP and cAMP (Table 1) [118, 136]. For example, inside-out vesicular studies using human erythrocytes suggest that

MRP5 is a high affinity transporter for cGMP and low affinity transporter of cAMP. In contrast, the opposite is true for MRP4, whereas MRP8 transports both cAMP and cGMP with moderate to low affinity [136-138]. In addition, MRP5 acts as a selective transporter of cGMP in pituitary cells [139] and pial arteriolar smooth muscle [140], and cGMP transport is inhibited when the membranous extract was incubated with an anti-MRP5 antibody [141]. Vesicular transport studies by Jedlitschky et al. [136] suggested that phosphodiesterases inhibitors, such as trequensin and sildenafil, effectively blocked cGMP and cAMP efflux, thereby raising the possibility that these latter compounds may work by interfering with MRP5. In support of this possibility, MRP5 is highly expressed in the tissues of urinogenital system [134], which also express high levels of phosphodiesterases. However, this role for MRP5 in the urinogenital system is yet to be proven.

MRP5, like MRP4, is a cyclic nucleotide organic anion transporter, mediating the efflux of a variety of organic anions, including certain monophosphate nucleotide metabolites such as cGMP and cAMP, and certain purine analogues [136]. Consequently, MRP5 is sometimes referred to as a “cyclic nucleotide efflux pump”. In addition, MRP5 preferentially extrudes unmethylated thionucleotides, whereas MRP4 appears to prefer methylated thio-ionosine monophosphate [118]. Moreover, vesicular transport studies indicate that the substrate profile of MRP5 also includes organic anions, such as *S*-(2, 4-dinitrophenyl) glutathione (DNP-SG) and GSH (Table 1) [118, 119, 129, 130]. A recent *in vitro*, *ex vivo* and *in vivo* study provided evidence that MRP5 can actively efflux and lower the permeability of the antiviral drug acyclovir and glaucoma drugs including bis(POM)-PMEA (adefovir) in the rabbit cornea [133].

McAleer et al. [130] initially reported that MRP5 ectopically overexpressed in human embryonic kidney cells did not confer resistance to anticancer drugs such as daunorubicin or cisplatin, whereas resistance was observed to certain metallic salts such as potassium antimonyl tartrate and cadmium chloride (Table 1). Nevertheless, elevated levels of MRP5 mRNA were observed in clinical lung cancer samples obtained following long-term treatment of patients with cisplatin [142]. MRP5 levels have been reported to be elevated in lung, colon, pancreatic and breast cancer samples and also in the heart following ischemic episodes [131, 132, 143]. Furthermore, in two *in vitro* studies, MRP5 mRNA was detected in non-small cell lung cancer cells following exposure to cisplatin and doxorubicin [142, 144]. In addition, subsequent *in vitro* studies have reported that MRP5 could confer resistance to several anticancer drugs, including cisplatin, purine analogues (such as 6-mercaptopurine and 6-thioguanine), pyrimidine analogues such as (gemcitabine, cytosine arabinoside (Ara-C) and 5-fluorouracil (5-FU)), the natural product doxorubicin and to anti-folate drugs (such as MTX), but not to *Vinca* alkaloids (such as vincristine) (Table 1) [98, 128, 129, 145, 146]. Further investigation is mandated to understand the role of MRP5 in mediating clinical resistance to antiretroviral- and cancer chemo-therapy.

MRP6/ABCC6

MRP6 (gene symbol *ABCC6*) was originally found by amplication of the 3'-end of the *ABCC6* gene in epirubicin resistant human leukemic cells [147]. The mouse and rat *Mrp6* orthologs show greater than 78% amino acid identity with human MRP6 [148]. The human *MRP6* gene is located on chromosome 16, immediately next to *MRP1* and consists of 31 exons spanning around 73 kb of genomic DNA [148]. Human MRP6 shares 45% amino acid identity with MRP1. The highest levels of *MRP6* mRNA and MRP6 protein expression are detected in the liver and kidney, although low levels have been detected in most other tissues, including the skin and retina [148-150].

Membrane vesicles prepared from *MRP6* transfected Chinese hamster ovary (CHO) cells have shown that MRP6 can mediate the *in vitro* transport of the prototypical GSH conjugates LTC₄ and DNP-SG but not estradiol glucuronide or cyclic nucleotides [151]. Similarly, insect cell membranes enriched with MRP6 supported transport of GSH conjugates but not glucuronide conjugates [152]. However, the relevance of these observations to the physiological or pharmacological function(s) of MRP6 is unclear.

MRP6 transfected CHO cells show low levels of resistance to a variety of anticancer drugs such as etoposide, teniposide, doxorubicin, daunorubicin, actinomycin D and cisplatin [151]. However, there is no evidence thus far which supports the role of MRP6 in clinical multidrug resistance.

MRP6/ABCC6 and Pseudoxanthoma Elasticum

Mutations in *MRP6* have been demonstrated to be the genetic basis of Pseudoxanthoma elasticum (PXE), a heritable connective tissue disorder that affects the elastic tissues primarily in the skin, eyes and cardiovascular system in the body [153-156]. Clinical manifestations of this disease include visual impairment, blood vessel rupture and myocardial infarction [157]. Histopathological abnormalities of the elastic fibers are manifested by the accumulation of the elastotic material in the skin and calcification of elastic structures [157, 158]. The symptoms of PXE have been recapitulated in an animal model by disruption of the *Mrp6* gene in mice [159, 160].

Several theories have been postulated to explain how mutations in a gene expressed primarily in the liver can alter the elastic structures in different tissues. One of these, the “metabolic hypothesis”, states that as a result of non-functional MRP6 pump in the liver, normal calcium/phosphate homeostatic conditions become deficient, resulting in the mineralization of elastic fibers [161, 162]. Recently, identification of a number of anti-mineralization factors that are necessary to be in balance with mineralization factors under homeostatic conditions supports this hypothesis. Anti-mineralization factors including fetuin A, osteopontin, and matrix Gla protein were identified through studies of *Mrp6* knockout mice, which show mineralization of elastic fibers [163-165]. More recently, it has also been proposed that tissue mineralization is due to the absence of a plasma factor (possibly a vitamin K precursor) from the basolateral hepatocyte membrane [166]. The mutation spectra of *MRP6* in PXE patients include nonsense, missense and frameshift mutations (Fig. 3B). The majority of mutations occur in NBD1 and NBD2, as well as the cytoplasmic loop between TM15 and TM16 (Fig. 3B) [167, 168].

MRP7/ABCC10

MRP7 (gene symbol *ABCC10*) was identified to have the lowest amino acid sequence identity (33-36%) when compared to other members of the MRP family [169]. Nevertheless, the MRP7 protein exhibits a membrane topology similar to that of MRP1, MRP2, MRP3, and MRP6, in that its 17 TM helices are arranged in three MSDs [169]. The structure and organization of the human *MRP7* gene, which consists of 22 exons and 21 introns, also differs significantly from other *MRP* genes [170]. *MRP7* mRNA is highly expressed in the colon, skin and testes, although it can be detected in other tissues [169].

To gain insight into the *in vivo* physiological and pharmacological roles of MRP7, a knockout strain of mouse model with a disrupted *Mrp7* gene was generated and it was reported that the *Mrp7*^{-/-} mice are fertile and appear healthy [171]. This null mouse model would be useful in determining the role of MRP7 *in vivo*, in the protection against toxicity associated with cancer chemotherapy. The substrate specificity and resistance profile of MRP7 have been examined in *MRP7*-transfected human embryonic kidney cells, which

indicates that MRP7 is a lipophilic anion transporter [172, 173]. MRP7 mediates the transport of glucuronate conjugates such as E₂17βG and to a lesser extent GSH conjugates such as LTC₄ [172]. It has been proposed that MRP7 (like other MRP family members) possesses a bipartite substrate binding pocket that can interact with anionic and lipophilic ligands. The transport of E₂17βG can be competitively inhibited by other organic anions such as LTC₄, glycolithocholate 3-sulfate, MK-571, and lipophilic agents such as cyclosporine A [172].

Resistance to docetaxel, paclitaxel, vincristine, and vinblastine mediated by MRP7 has been reported in an *in vitro* study [173]. MRP7 is also able to confer resistance to nucleoside-based agents such as Ara-C and gemcitabine, and to the microtubule stabilizing agent epothilone B [174]. Vincristine-treated human and mouse salivary gland adenocarcinoma cells express elevated levels not only of *MDR1/Mdr1* and *MRP1/Mrp1*, but also *MRP7/Mrp7* [175]. In addition, *MRP7* expression has been detected in non-small cell lung cancer cells after the exposure to either paclitaxel [176] or vinorelbine [177]. However, there is no clinical study reported thus far to ascertain the role of MRP7 in clinical drug resistance.

MRP8/ABCC11

MRP8 (gene symbol *ABCC11*) was first identified to be highly expressed in breast cancer through a gene prediction program and EST database mining [178]. The full length human MRP8 transporter is predicted to be 1382 amino acids in length and similar to MRP4 and MRP5, MRP8 is a short MRP with 12 TM helices [86, 178, 179]. Human *MRP8* is located on chromosome 16q12.1 and unlike other MRP family members, no orthologous genes have been found in mammals except for primates (Table 1) [179, 180]. Conflicting reports exist regarding the expression of *MRP8* mRNA transcripts, with widespread expression reported by some research groups [179, 180], while others suggest that expression of the transcripts is limited, with highest levels in the liver, brain, placenta, breasts and testes [178]. Multiple splice forms of the *MRP8* transcript have been described and this may account, at least in part, for the reported variations in transcript levels [179]. Indeed, it is difficult at present to correlate mRNA transcript levels with tissue MRP8 protein levels. Therefore, additional protein expression studies are required to determine MRP8 tissue expression, and how they relate to the multiple splice forms of *MRP8*. MRP8 protein has been detected in axons of the human central and peripheral nervous system neurons, and it has been proposed that it plays a role in the efflux of neuromodulatory steroids such as dehydroepiandrosterone 3-sulphate (DHEAS) [181].

Using membrane vesicles prepared from transfected cells, MRP8 has been shown to transport a wide range of compounds, including cyclic nucleotides cGMP and cAMP (similar to MRP4 and MRP5), lipophilic anions including natural and synthetic glutathione conjugates such as LTC₄ and DNP-SG, estradiol glucuronide, sulphated conjugates such as DHEAS and estrone sulphate, glucuronidated steroids and folic acid (Table 1) [138].

Significant levels of *MRP8* transcripts have also been reported in breast cancer samples [178]. Guo et al. analyzed the drug resistance characteristics of MRP8 using *MRP8* transfected LLC-PK1 cells [182]. In this system, MRP8 conferred resistance to antimetabolites including such as PMEA, MTX, Ara-C and 5-fluorouracil (Table 1) [182]. The same group recently reported that the high expression of MRP8, but not of MRP4 and MRP5, is significantly associated with low probability of overall survival in AML patients, suggesting that MRP8 may be a predictive biomarker for the treatment outcome in AML [183]. However, the levels and activity of MRP8 in human tissues have not yet been established. Further studies are needed to provide insight into whether MRP8 plays any role in clinical multidrug resistance.

MRP8/ABCC11 and Dry/wet Earwax

Insight into a physiological role for MRP8 in cerumen (earwax) secretion by the ceruminous apocrine glands was revealed by the identification of a single nucleotide polymorphism, 538G>A (Gly180Arg) in the *MRP8* gene, which was associated with the production of wet rather than dry earwax [184]. Individuals with the AA genotype, which is common in East Asian populations, secrete dry earwax, while individuals with the GG or GA genotype (common in individuals of European and African descent) produce wet earwax [184]. The authors thus speculated that MRP8 might be involved in the secretion of the aliphatic or aromatic hydrocarbon constituents in ear wax [184]. The MRP8 protein produced by the dry earwax population (AA genotype) lacks *N*-linked glycosylation and localizes to the endoplasmic reticulum (ER) and undergoes proteosomal degradation presumably because trafficking to the plasma membrane has been disrupted possibly because of misfolding [185]. In addition, phenotypic analyses has suggested a positive association between the wet earwax type (individual with GG or GA genotype), and axillary osmidrosis (armpit secretions of fetid sweat), colostrums secretion, and a risk of developing breast cancer [185-188]. It is suggested that the non-conservative mutation at amino acid 180 in TM1 of *MRP8*, which introduces a charged residue may result in a change in the conformation of MRP8 transporter. In membrane vesicles studies, the G180R mutant of *MRP8* was unable to transport cAMP, again supporting the notion that a deficiency of MRP8 transport activity is responsible for the dry earwax type.

MRP9/ABCC12

MRP9 (gene symbol *ABCC12*) is located next to *MRP8* on chromosome 16q12.1, 20, oriented in a tail-to-head position, suggesting that *MRP9* most likely arose from a gene duplication event [179]. The longest mRNA transcript of *MRP9* is predicted to encode a protein of 1359 amino acids [179]. Similar to *MRP8*, multiple splice variants of *MRP9* have also been characterized [179, 189]. The murine ortholog of *Mrp9* has been described and *Mrp9* transcripts are expressed in the ovary, brain, breast, prostate and testis [189-192]. It has been proposed that MRP9 may play a role during the latter part of the male meiotic prophase, spermatid development or in sperm function, as full-length *Mrp9* appears to be expressed only in testicular germ cells and sperm of mouse and boar [193]. The ectopic expression of a full-length human *MRP9* transcript in human embryonic kidney cells gives rise predominantly to a non-glycosylated, 150 kDa protein in the endoplasmic reticulum [193]. Until now, the physiological role of MRP9 and its involvement in cancer chemotherapy have not been reported.

Summary

The nine MRP/ABCC members have considerable differences in membrane localization, tissue expression, substrate specificities and proposed pharmacological and physiological functions. Most of the MRP members are involved in translocation or conjugation of a variety of structurally diverse endogenous (such as organic anion conjugates i.e. LTC₄, PGE₂, E₂17βG) or xenobiotic (such as therapeutic drugs and their metabolites) substrates (see Table 1). Involvement of GSH transport is a characteristic feature of some of the MRP family members. MRPs 1-3 confer resistance to hydrophobic anions such as several natural compounds and MTX, whereas MRPs 4, 5 and 8 efflux cyclic nucleotides. At least, MRPs 1-8 have shown to confer resistance to variety of amphipathic anticancer drugs in *in vitro* studies. MRPs 1-7 knock out mouse models are available, and will help further to clarify the *in vitro* substrate specificity. Mutations in *MRP* gene is related to certain hereditary abnormalities such as mutations of *MRP2* causes Dubin-Johnson syndrome, mutations of *MRP6* causes Pseudoxanthoma elasticum and mutations of *MRP8* is linked to wet rather than a dry ear wax type. With the presently available preclinical and scarce clinical studies

one could expect that modulation of MRP members may affect disposition and elimination of variety of clinical drugs including anticancer drugs. Further understanding through crystallographic studies or clinical studies directed more towards specific MRP member could have important implications in management of anticancer agents and other diseases such as inflammatory disease.

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Figure 1. Relatedness of the human MRP/ABCC drug transporters

A dendrogram illustrating the relative similarity of the human MRP/ABCC proteins, based on ClustalW alignments and generated using Phylip's Drawgram (<http://workbench.sdsc.edu>). The gene symbols for the MRPs are indicated in parentheses and in italics. The genetic disorders associated with mutations in several *ABCC* genes are also indicated (DJS, Dubin-Johnson syndrome; PXE, *Pseudoxanthoma elasticum*; EWX, dry/wet earwax phenotype). This figure was courtesy of Dr. Susan P.C. Cole.

Figure 2. Domain structure and cellular localization of the MRPs

The long MRPs (MRP1, MRP2, MRP3, MRP6 and MRP7) contain three MSDs (with 17 transmembrane helices) and the NH₂-terminus (N) is extracellular; the short MRPs (MRP4, MRP5, MRP8) contain just two MSDs (with 12 transmembrane helices) and the NH₂-terminus (N) is intracellular. The two cytoplasmic nucleotide binding domains (NBD1, NBD2) found in all ABC proteins are also shown. Endothelial and epithelial cells can grow in a polarized fashion, creating distinct apical and basolateral domains with different biochemical properties. Shown below the domain structures of the long and short MRPs are cartoon drawings illustrating the membrane localization of the various MRPs in such polarized cells. MRP6 is localized to the basolateral membranes of polarized cells, whereas the membrane localization of MRP7 and MRP9 remains to be elucidated. This figure was courtesy of Dr. Susan P.C. Cole.

Figure 3. Cartoon structures showing the location of (A) missense mutations in MRP2 (*ABCC2*), associated with Dubin-Johnson syndrome and (B) missense mutations in MRP6 (*ABBC6*) associated with Pseudoxanthoma elasticum.

Table 1

Summary of some properties of the human MRP family.

Common name	Alternate name/Symbol	Chromosomal location (transcript length)	Amino acids	Tissue expression	Physiological substrates	Resistance profile (anticancer drugs)
MRP1	ABCC1	16p13.12 (5927 bp)	1531	Ubiquitous	LTC ₄ , E ₃ 17βG, sulfated bile acids, folic acid, bilirubin, GSH conjugates	Anthracyclines, camptothecins, vinca alkaloids, antifolate neoplastics (MTX, edatrexate), etoposide, irinotecan, SN-38
MRP2	ABCC2/cMOAT	10q24.2(4930 bp)	1545	Liver, kidney, intestine	Bilirubin conjugates, LTC ₄ , LTD ₄ , LTE ₄ , GSH	Anthracyclines, camptothecins, vinca alkaloids, MTX, etoposide, cisplatin, irinotecan
MRP3	ABCC3/MOAT-D	17q21.33 (5176 bp)	1527	Small intestine, pancreas, colon, kidneys, placenta, adrenal gland	Taurocholate, glycocholate, cholate, E ₂ 17βG, LTC ₄	MTX, teniposide, etoposide
MRP4	ABCC4/MOAT-B	13q32.1 (5871 bp)	1325	Prostate, testis, ovary, lung, hepatocytes, intestine, pancreas	cGMP, cAMP, PGE1, PGE2, E ₂ 17βG, DHEAS, GSH, and GSH conjugated bile acid, LTB ₄ and LTC ₄	MTX, 6-TG, 6-MP, SN-38, irinotecan, topotecan, PMEA, AZT
MRP5	ABCC5/MOAT-C	3q27.1 (5851 bp)	1437	All major tissues	cGMP, cAMP, DNP-SG, GSH, DNP-SG	5-FU, 6-MP, 6-TG, MTX, cisplatin, PMEA, AZT, daunorubicin, DOX, gemcitabine, Ara-C
MRP6	ABCC6/MOAT-B/PXE	16p13.12 (5111 bp)	1503	Kidney, liver	LTC ₄ , DNP-SG	DOX, daunorubicin, etoposide, teniposide, cisplatin, actinomycin-D
MRP7	ABCC10	6p21.1 (5118 bp)	1492	Most tissues	E ₂ 17βG, LTC ₄	Paclitaxel, docetaxel, vincristine, vinblastine, Ara-C, gemcitabine, epothilone-B
MRP8	ABCC11	16q12.1 (4576 bp)	1382	Testis, breasts	cGMP, cAMP, E ₂ 17βG, DHEAS, LTC ₄ , bile acids, estrone 3-sulfate, DNP-SG, folic acids	5-FU, MTX, Ara-C, PMEA, ddC
MRP9	ABCC12	16q12.1 (5168 bp)	1356	Testis, breasts, ovary, brain, skeletal muscles	Unknown	Unknown

Ara-C, arabinofuranosyl cytidine; AZT, azathioprine; ddC, 2',3'-dideoxycytidine; DHEAS, dehydroepiandrosterone sulphate; DNP-SG, S-(2,4-dinitrophenyl)glutathione; DOX, doxorubicin; E₂17βG, estradiol 17β-D-glucuronide; GSH, glutathione; LTC₄, leukotriene C₄; MTX, methotrexate; 6-MP, 6-mercaptopurine; PMEA, 9-((2-phosphonylmethoxy)ethyl)-adenine; 6-TG, 6-thioguanine