Chapter 2
ABC Family Transporters

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Abstract The transport of specific molecules across lipid membranes is an essential function of all living organisms. The processes are usually mediated by specific transporters. One of the largest transporter families is the ATP-binding cassette (ABC) family. More than 40 ABC transporters have been identified in human, which are divided into 7 subfamilies (ABCA to ABCG) based on their gene structure, amino acid sequence, domain organization, and phylogenetic analysis. Of them, at least 11 ABC transporters including P-glycoprotein (P-GP/*ABCB1*), multidrug resistance-associated proteins (MRPs/ABCCs), and breast cancer resistance protein (BCRP/*ABCG2*) are involved in multidrug resistance (MDR) development. These ABC transporters are expressed in various tissues such as the liver, intestine, kidney, and brain, playing important roles in absorption, distribution, and excretion of drugs. Some ABC transporters are also involved in diverse cellular processes such as maintenance of osmotic homeostasis, antigen processing, cell division, immunity, cholesterol, and lipid trafficking. Several human diseases such as cystic fibrosis, sitosterolemia, Tangier disease, intrahepatic cholestasis, and retinal degeneration are associated with mutations in corresponding transporters. This chapter will describe function and expression of several ABC transporters (such as P-GP, BCRP, and MRPs), their substrates and inhibitors, as well as their clinical significance.

Keywords Multidrug resistance · P-glycoprotein · Breast cancer resistance protein · Multidrug resistance-associated proteins · ABC gene-related disease

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2.1 Introduction

2.1.1 General Properties of ABC Transporters

The transport of specific molecules across lipid membranes is an essential function of all living organisms. The processes are usually mediated by specific transporters. One of the largest transporter gene families is the ATP-binding cassette (ABC) family. The ABC transporters bind ATP and use the energy to drive the transport of various molecules including sugars, amino acids, metal ions, peptides, proteins, hydrophobic compounds, and their metabolites across the plasma membrane as well as intracellular membranes of the endoplasmic reticulum, peroxisome, and mitochondria. ABC transporters are integral membrane proteins. Human P-glycoprotein (P-GP) is first described and identified as the cause of cytotoxic drug resistance by Juliano and Ling in 1976. Subsequently, a series of ABC transporters have been coined and identified (Dean et al. 2001).

To date, 49 ABC transporter subtypes, including a pseudogene, have been identified in humans, and they are further divided into 7 subfamilies (ABCA to ABCG) based on their gene structure, amino acid sequence, domain organization, and phylogenetic analysis (Dean et al. 2001). ABC transporters identified in humans are primarily located on the plasma membrane, where they export a variety of diverse drugs, drug conjugates, and metabolites out of cells. Of them, at least 11 ABC superfamily transporters including P-GP (/*ABCB1*), multidrug resistance-associated proteins (MRPs/*ABCCs*), and breast cancer resistance protein (BCRP/*ABCG2*) are involved in multidrug resistance (MDR) development (Schinkel and Jonker 2012; Slot et al. 2011) (Table 2.1). These ABC transporters are also expressed in various tissues such as the liver, intestine, kidney, and brain, playing important roles in absorption, distribution, and excretion of drugs. Some ABC

Table 2.1 Properties of several human ABC drug efflux transporters

Protein	Gene name	Alternative names	Size (AA)	Polarized localization
MDR1 (P-GP)	*ABCB1*	PGY1, GP170	1280	Apical
MDR2	*ABCB4*	PGY3, MDR3	1279	Apical
BESP	*ABCB11*	/	1321	Apical
MRP1	*ABCC1*	MRP	1531	Basolateral
MRP2	*ABCC2*	cMOAT, cMRP	1545	Apical
MRP3	*ABCC3*	MOAT-D, MOAT-2	1527	Basolateral
MRP4	*ABCC4*	MOAT-B	1325	Basolateral, apical[a]
MRP5	*ABCC5*	MOAT-C, pABC11	1437	Basolateral, apical[a]
MRP6	*ABCC6*	/	1503	Apical
MRP7	*ABCC10*	/	1492	Apical?
MRP8	*ABCC11*	/	1382	Apical
MRP9	*ABCC12*	/	1356	?
BCRP	*ABCG2*	MXR, ABCP	655	Apical

[a]was expressed at apical membrane of the brain microvessel endothelial cells

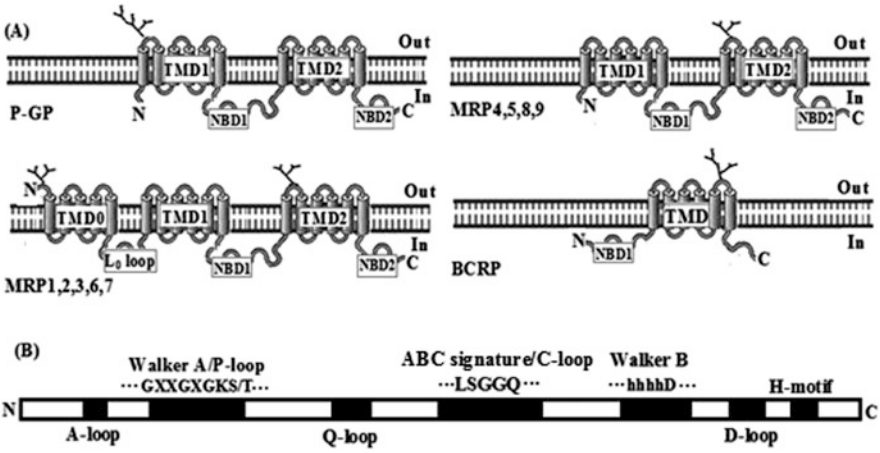

Fig. 2.1 (**a**) Secondary structure models of drug efflux transporters of the ATP-binding cassette family. TMD, transmembrane domain; NBD, nucleotide-binding domain. "In" and "out" are termed as cytoplasmic and extracellular compartments, respectively. (**b**) Linear arrangement and residues of the canonical elements of the NBD. "h" is any hydrophobic residue

transporters are also involved in diverse cellular processes such as maintenance of osmotic homeostasis, antigen processing, cell division, immunity, cholesterol, and lipid trafficking (Bojanic et al. 2010; Cole 2014; Dean et al. 2001; Eckford and Sharom 2009; Gadsby et al. 2006; Kooij et al. 2009; Tarling et al. 2013; Yvan-Charvet et al. 2010). Several human diseases related to genes as cystic fibrosis, sitosterolemia, Tangier disease, intrahepatic cholestasis, retinal degeneration, and drug response are associated with mutations in corresponding transporters.

The functional proteins typically contain two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs) (Fig. 2.1). The TMDs consist of six transmembrane-spanning α-helices (TMs) and provide the specificity for the substrate. The NBDs are highly conserved throughout the different ABC transporters. The NBDs are located in the cytoplasm and transfer the energy to transport the substrate across the membrane. Several motifs have been identified in each of the NBD including the Walker A, Walker-B, A-loop, H-loop, D-loop, Q-loop, and the signature motif "LSSGQ" consensus sequences. Each NBD can be divided into two subdomains, a catalytic core domain and an α-helical domain. The catalytic core domain contains conserved motifs, the Walker A motif (or phosphate-binding P-loop) and the Walker B motif, for the binding and hydrolysis of ATP. The Walker A is a highly conserved glycine-rich motif (G-X-X-G-X-G-KS/T) and interacts with the phosphate groups of the nucleotide, while Walker B motif has the consensus sequence h-h-h-h-D (where "h" is any hydrophobic residue) and its glutamate residue acts as a general base to activate a water molecule for nucleophilic attack at the γ-phosphate of ATP. The α-helical domain contains the motif LSGGQ known as the ABC signature motif (or C-loop), which is located just upstream of the Walker B site and involves the binding of the nucleotide. The ABC signature motif is

specific to ABC transporters and distinguishes them from other ATP-binding proteins. The NBDs are arranged "head-to-tail" that form the full composite ATP-binding and hydrolysis site upon dimerization via interactions with the D-loop. The ATP-binding site is formed from the Walker A and Walker B motifs of one subunit and the Q-loop of the other subunit, so that two ATP molecules can bind and hydrolyze. In the absence of nucleotides, the NBDs are separated, open conformation, but upon ATP binding they come close together to sandwich the ATP molecules and form the complete interface. ATP hydrolysis disrupts the dimer interface and releases ADP and inorganic phosphate (Beis 2015).

Effective coupling of transport to ATP binding requires the transmission of the molecular motion from the NBDs to the TMDs. The TMDs interact with the NBDs through a coupling helix located in the cytoplasmic loops of the TMD. The structure of the coupling helix is conserved but its sequence varies between the different transporters. The coupling helix of P-GP is located between TM4 and TM5 (Aller et al. 2009).

The mechanisms of ABC efflux transporter-mediated substrate translocation are not fully understood, but the "ATP switch" model seems to explain the functional "choreography" of ABC transporters (Al-Shawi 2011; Chen et al. 2016; George and Jones 2012; Higgins and Linton 2004). The model has three consistent elements (Al-Shawi 2011). (i) Binding of substrate to the TMDs of the apo form in the "high-affinity inward-facing orientation" initiates the transport cycle. (ii) ATP binding induces the formation of the closed nucleotide sandwich structure. ATP acts as molecular glue that holds the two NBDs together, forming two NBSs (nucleotide-binding sites) at the interface of the two NBD. The binding energy gained by the NBDs/NBSs is transmitted to the TMDs which then change their access to the "low-affinity outward-facing orientation" on the other side of the membrane. The gate to the inside is closed, and the gate to the outside of the membrane is opened. The affinity of the transported entity changes (switches) from high affinity (low chemical potential of substrate) to low affinity (high chemical potential). The ATP-binding step can be considered as the power stroke in which the chemical potential of the transported entity changed. (iii) ATP hydrolysis leads to the formation of extra negative charge, thus opening the closed nucleotide sandwich structure. The opening of the nucleotide sandwich structure facilitates Pi release and ADP dissociation, which in turn allows the TMDs and access gates to reset to the high-affinity orientation on the original side of the membrane (Fig. 2.2).

ABC transporters are organized either as full transporters (TMD1-NBD1-TMD2-NBD2) or as half transporters (TMD-NBD). The latter must form either homodimers or heterodimers to constitute a functional transporter. Based on their predicted two-dimensional structure in membranes, ABC transporters can be divided into four classes (Fig. 2.1). Typically, P-GP has two NBDs and TMDs. First extracellular loop in P-GP is heavily *N*-glycosylated. The same overall architecture is found in MRP4 and MRP5, but N-linked glycosylation occurs most likely on the fourth extracellular loop. MRP1, MRP2, and MRP3 have the same basic structure as MRP4 and MRP5, but in addition they have an N-terminal extension consisting of five TMs which is the so-called terminal TM (TMD0),

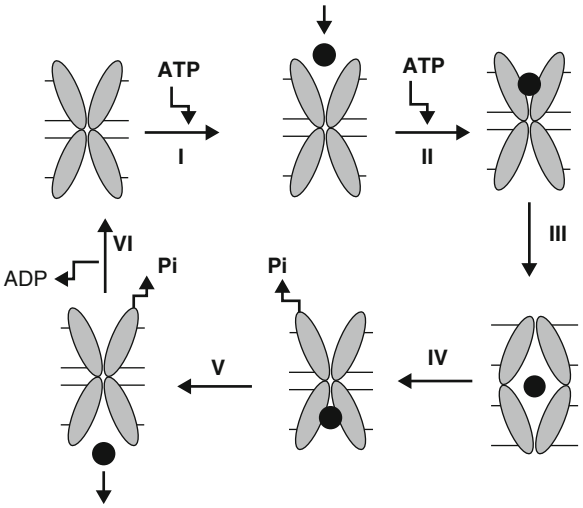

Fig. 2.2 Switch models for catalytic cycle of ATP binding and hydrolysis in the ABC transporter NBD dimer: Step I, resting state. The ATP-binding cassette monomers are separated in the absence of nucleotide. From Step I to Step II, the monomers are loaded processively with ATP, triggering the closure of the interface to form the sandwich dimer (Step III). Steps IV and V depict processive hydrolysis of ATP in each site followed by sequential release of Pi and ADP, returning the dimer to the nucleotide-free open state during Step VI, completing the cycle

which linked to the core of the molecule by a L_0-loop. As a consequence, the N-terminus of these proteins is located extracellularly and also glycosylated in MRP1. BCRP belongs to half transporter, only possessing one NBD and TMD. *N*-glycosylation occurs in the last extracellular loop. All these ABC transporters possess to a greater or lesser extent extracellular *N*-glycosylation branches. Although the *N*-glycosylation seems not to be necessary for the basic transport function of these transporters, *N*-glycosylation probably has an important biological role in these proteins, helping in stabilizing membrane insertion.

2.1.2 Overview of Human ABC Gene Subfamilies

2.1.2.1 ABCA Family

The human ABCA subfamily comprises 12 protein-coding genes, A*BCA1–ABCA13*, with "ABCA11" representing a transcribed pseudogene. All ABCA proteins are full-sized transporters. According to their predicted primary structure, ABCA transporters are polypeptides ranging from 1543 amino acids (*ABCA10*) to 5058 amino acids (*ABCA13*) in size with a calculated molecular weight between 176 and 576 kDa. Most *ABCA* transporters show a broad tissue specificity. But *ABCA4* is mainly expressed in the eye, and *ABCA13* is detectable only in a small

variety of tissues. These transporters evolve from a common ancestor gene and can be further divided into two subgroups (Piehler et al. 2012; Tarling et al. 2013). Subgroup I, called "*ABCA6*-like" transporters, includes *ABCA5*, *ABCA6*, *ABCA8*, *ABCA9*, and *ABCA10*. These transporters form a compact gene cluster on chromosome 17q24 and are characterized by a strikingly high, mutual amino acid sequence identity and a relatively smaller size (between 1543 amino acids and 1642 amino acids) than other *ABCA* transporters. Group II include the remaining seven *ABCA* transporters (*ABCA1*, *ABCA2*, *ABCA3*, *ABCA4*, *ABCA7*, *ABCA12*, and *ABCA13*), which are dispersed on six chromosomes. Most of ABCA transporters mediate transport of lipids or lipid-related compounds. Several ABCA transporters are linked to monogenetic diseases in humans such as *ABCA1* to high-density lipoprotein deficiency and Tangier disease (Puntoni et al. 2012; Zyss et al. 2012), *ABCA3* to neonatal surfactant deficiency (Albrecht and Viturro 2007; Peca et al. 2015; Wambach et al. 2014), *ABCA4* to Stargardt disease (Molday et al. 2009), and *ABCA12* to Harlequin ichthyosis (Scott et al. 2013; Thomas et al. 2006). The ABCA transporters are also involved in more complex diseases like atherosclerosis (*ABCA1*), pediatric interstitial lung diseases (*ABCA3*), age-related macular degeneration (*ABCA4*), and Alzheimer disease (*ABCA1*, *ABCA2*, *ABCA7*, and *ABCA5*) (Fu et al. 2015; Li et al. 2015; Piehler et al. 2012). A report also demonstrated involvement of *ABCA2* and *ABCA3* in MDR development (Rahgozar et al. 2014).

2.1.2.2 ABCB Family

The human ABCB transporter subfamily consists of 11 members; among them, 4 full transporters and 7 half transporters are currently identified. *ABCB1* (P-GP/MDR1/PGY1) is the first human ABC transporter cloned and characterized through its ability to confer a multidrug resistance phenotype to cancer cells. In addition to drug-resistant cancer cell, P-GP is widely expressed in various tissues such as the intestine, liver, and brain, where it mediates drug transport. *ABCB4*, another member of multidrug-resistant transporters, is mainly expressed on the apical membrane of hepatocytes and functions to transport phosphatidylcholine from hepatocytes into the bile canaliculus, whose mutations are attributed to intrahepatic cholestasis type 3. *ABCB11*, a bile salt export pump (BSEP), is responsible for bile acid-dependent bile flow at the apical membrane of hepatocytes, whose mutations are linked to intrahepatic cholestasis type 2. *ABCB2* and *ABCB3* are called antigen peptide transporter (TAP) 1 and TAP2, respectively. The *ABCB2* and *ABCB3* proteins are half transporters with an extra N-terminal transmembrane domain consisting of four TMs. *ABCB2* and *ABCB3* play important roles in the adaptive immune system. They form a heterodimer to transport antigenic peptides from the cytosol to the lumen of the endoplasmic reticulum (Hinz and Tampé 2012; Seyffer and Tampé 2015). *ABCB9*, TAP-like transporter, is localized to lysosomes and transports peptides from the cytosol into the lysosome coupled to ATP hydrolysis (Zollmann et al. 2015). The remaining four half transporters, *ABCB6*, *ABCB7*, *ABCB8*, and *ABCB10*, are localized to the mitochondria, where they are involved in iron metabolism and

transport of Fe/S protein precursors. *ABCB6* is the first ABC transporter located in the outer mitochondrial membrane and oriented to facilitate porphyrin importer (Ulrich et al. 2012) while *ABCG2*, located at the plasma membrane, provides a mechanism to remove excess porphyrins. Thus, the *ABCB6* and *ABCG2* coordinately modulate porphyrin concentrations under normal physiological and pathological condition, inferring important roles of *ABCB6* in keeping homeostasis of hemoproteins (Krishnamurthy and Schuetz 2011; Lynch et al. 2009). *ABCB6* is also located in plasma membrane of red cells (Fukuda et al. 2016) and mediates the plasma membrane export of porphyrins in red cells. The *ABCB6* mutations are linked to severity of hereditary porphyria (Fukuda et al. 2016) or familial pseudohyperkalemia (Andolfo et al. 2016).

2.1.2.3 ABCC Family

The ABCC family forms the largest gene family, consisting of 13 subtypes. Except for *ABCC13*, which is a pseudogene, the ABC transporters coded by ABCC genes are termed as multidrug resistance-associated proteins (MRPs). The proteins encoded by *ABCC1*, *ABCC2*, *ABCC3*, *ABCC4*, *ABCC5*, *ABCC6*, *ABCC10*, *ABCC11*, and *ABCC12* genes are the MRP members called MRP1 to MRP9, respectively. Among the nine ABCC proteins, four (MRP4, MRP5, MRP8, and MRP9) have a typical ABC structure with two TMDs and two NBDs. Others (MRP1, MRP2, MRP3, MRP6, and MRP7) have an extra domain (TMD_0). Similarly to P-GP proteins, MRPs play an important role in MDR development (Slot et al. 2011). Several ABCC transporters are linked to genetic diseases such as *ABCC2* to Dubin-Johnson syndrome and *ABCC7* to cystic fibrosis (Dean et al. 2001).

2.1.2.4 ABCD Family

The ABCD subfamily contains four genes that encode four half transporters *ABCD1* (adrenoleukodystrophy protein (ALDP)), *ABCD2* (adrenoleukodystrophy-related (ALDR) protein), *ABCD3* (70 kDa peroxisomal membrane protein (PMP70)), and *ABCD4*, respectively. *ABCD1*, *ABCD2*, and *ABCD3* are extensively located in peroxisomes. They need to dimerize to constitute full, active transporters. ABCD transporters have distinct but overlapping specificities for different acyl-CoA esters. Most hydrophobic C24: 0-CoA and C26:0-CoA esters are preferentially transported by *ABCD1*, whereas C22:0-CoA, C22:6-CoA, and C24:6-CoA are preferentially transported by *ABCD2*. Substrates such as long-chain unsaturated acyl-CoAs, 2-methyl branched-chain acyl-CoAs including pristanoyl-CoA, and long-chain dicarboxylic CoA esters are preferentially transported by *ABCD3*. *ABCD1* is linked to a genetic disease, X-linked adrenoleukodystrophy (X-ALD) (Dean et al. 2001). *ABCD4* seems to be mainly expressed endoplasmic reticulum (Kawaguchi et al. 2016). The translocation of *ABCD4* from the endoplasmic reticulum to lysosomes requires, at least in part, the lysosomal membrane protein LMBD1, where *ABCD4*

mediates intracellular processing of vitamin B12 (Coelho et al. 2012; Deme et al. 2014). Mutations in *ABCD4* gene cause a new inborn error of vitamin B12 metabolism (Coelho et al. 2012; Kim et al. 2012; Takeichi et al. 2015).

2.1.2.5 ABCG Family

The ABCG family consists of five half transporters: *ABCG1*, *ABCG2*, *ABCG4*, *ABCG5*, and *ABCG8*. *ABCG1*, *ABCG4*, *ABCG5*, and *ABCG8* are involved in the ATP-dependent translocation of steroids and lipids, while *ABCG2* has been identified as a multidrug transporter that confers drug resistance on cancer cells. To become functionally active, they form a homodimer (*ABCG1*, *ABCG2*, and *ABCG4*) or an obligate heterodimer (*ABCG5* and *ABCG8*). The members of ABCG family, except *ABCG2*, play significant role in the efflux transport of cholesterol. Both *ABCG1* and *ABCG4* facilitate the efflux of excess cholesterol to high-density lipoprotein, a key player in the reverse cholesterol transport from macrophage to the liver. High expression of *ABCG4* was found in non-small cell lung cancer (NSCLC) tissues but not in normal lung tissues. The high *ABCG4* expression was associated with a poor prognosis in patients with NSCLC treated with cisplatin-based chemotherapy, inferring involvement of *ABCG4* in MDR development (Yang et al. 2015). *ABCG5* and *ABCG8*, mainly expressed in the liver and intestine, mediate the biliary and intestinal excretion of cholesterol. In addition, *ABCG5* and *ABCG8* also mediate the biliary excretion of phytosterols and intestinal efflux to prevent their accumulation. Mutations in *ABCG5* and *ABCG8* are linked to sitosterolemia (Hazard and Patel 2007).

2.2 P-Glycoprotein (P-GP/ABCB1)

2.2.1 General Properties and Distribution of P-GP

P-glycoprotein (P-GP/*ABCB1*), first discovered by Julian and Ling in drug-resistant cells, is a 170 kDa plasma membrane protein and is possibly the best studied ABC drug efflux transporter. Owing to broad substrate specificity of P-GP, the cell-expressed P-GP displays cross-resistance to many different cytotoxic drugs, termed as multidrug resistance (MDR). Human P-GP is coded by MDR1 gene (*ABCB1*). Rodents have two drug-transporting P-GP homologues, Mdr1a (encoded by *Abcb1a*) and Mdr1b (encoded by *Abcb1b*), which share approximately 85% amino acid identity with each other and > 80% amino acid identity with human P-GP. P-GP consists of two half transporters, each has a TMD and BND. The two half transporters share only 43% of their amino acid sequence identity. P-GP exports its substrate out of cell using energy derived from ATP hydrolysis. The stoichiometry is 0.3~3 ATP molecules per substrate molecule (Agnani et al. 2011; Eytan et al. 1996). In mammals, particularly in humans, P-GP is highly expressed in specific

organs including the intestine, liver, kidney, brain, testis, placenta, and eye, which is consistent with its role in general detoxification and in establishment of blood-brain, blood-testis, fetal-maternal, and blood-retinal barriers. P-GP is a promiscuous transporter of hydrophobic substrates or drugs such as colchicine, doxorubicin, and vinblastine, in turn, affecting the activity/toxicity of these drugs**.** P-GP is also expressed in many hematopoietic cell types such as $CD34^+$ stem cells, c-kit^+ stem cells, $CD56^+$ natural killer cells, and $CD8^+$ cytotoxic T cells, inferring roles of P-GP in immunomodulation (Kooij et al. 2009, 2010; Picchianti-Diamanti et al. 2014; Diamanti et al. 2011). Moreover, P-GP is also highly expressed in the mitochondrial membrane (Solazzo et al. 2006; Zhang et al. 2012) and nuclear (Zhang et al. 2012) of MDR-positive cells, where it pumps out anticancer drugs from mitochondria or nuclei into cytosol, which also implicates drug resistance development.

2.2.2 Substrates and Inhibitors of P-GP

P-GP has a very wide substrate spectrum mediating the export of a variety of drugs including chemotherapeutic drugs, HIV protease inhibitors, immunosuppressive agents, antiarrhythmics, calcium channel blockers, analgesics, antihistamines, antibiotics, natural products, fluorescent dyes, and pesticides (Table 2.2).

There are few common structural characters about P-GP substrates. The only common character identified so far in all P-GP substrates is their amphipathic nature. This may have to do with the mechanism of drug translocation by P-GP: it has been postulated that intracellular P-GP substrates first have to insert into the inner hemileaflet of the cell membrane, before being "flipped" to the outer hemileaflet or perhaps being extruded directly into the extracellular medium by P-GP. Only amphipathic molecules would have the proper membrane insertion properties.

Transport of P-GP-mediated substrates may be inhibited by certain compounds. These compounds are termed as "reversal agents" or "P-GP inhibitors." These inhibitors may inhibit function of P-GP via disrupting the hydrolysis of ATP, altering P-GP expression, or reversible/irreversible competition for a binding site. One of the most common mechanisms displayed by classical P-GP inhibitors is to compete with drug-binding sites. Most inhibitors, such as verapamil, quinidine, and cyclosporin A, are themselves P-GP substrates, suggesting that they act as competitive inhibitors. Some inhibitors are poorly transported by P-GP, indicating that they probably inhibit P-GP function via other mechanisms. On the basis of their specificity, affinity, and toxicity, P-GP inhibitors are categorized into three generations (Table 2.3).

The first general P-GP inhibitors, such as verapamil, cyclosporin A, vincristine, reserpine, quinidine, tamoxifen, and trifluoperazine, are relatively poor P-GP inhibitors in vivo, requiring high concentrations to inhibit P-GP. These P-GP inhibitors are also P-GP substrates. In addition, they frequently have their own pharmacological effects and may cause severely unwanted effects on patient. Therefore, these inhibitors are not suitable for clinical applications as inhibitor P-GP drugs. It is noteworthy

Table 2.2 Some clinically relevant transported substrates of P-GP

Analgesics	Asimadoline, fentanyl, morphine, pentazocine
Anticancer drug	5-fluorouracil, actinomycin D, bisantrene, chlorambucil, cytarabine, daunorubicin, docetaxel, doxorubicin, epirubicin, etoposide, gefitinib, hydroxyurea, irinotecan, methotrexate, mitomycin C, mitoxantrone, paclitaxel, tamoxifen, teniposide, topotecan, vinblastine, vincristine, imatinib, lapatinib, nilotinib
Antibiotics	Cefoperazone, ceftriaxone, clarithromycin, doxycycline, erythromycin, gramicidin A, gramicidin D, grepafloxacin, itraconazole, ketoconazole, posaconazole, levofloxacin, rifampicin, sparfloxacin, tetracycline, valinomycin
Antihistamines	Cimetidine, fexofenadine, ranitidine, terfenadine
Calcium channel blockers:	Azidopine, bepridil, diltiazem, felodipine, nifedipine, nisoldipine, nitrendipine, tiapamil, verapamil
HIV protease inhibitor	Saquinavir, ritonavir, nelfinavir, indinavir, lopinavir, amprenavir, maraviroc
Neuroleptics	Chlorpromazine, phenothiazine, trifluoperazine
Antiarrhythmics	Loperamide, amiodarone, lidocaine, propafenone, quinidine
Immunosuppressant	Cyclosporin A, cyclosporin H, sirolimus, tacrolimus, everolimus
Corticoids	Dexamethasone, hydrocortisone, corticosterone, triamcinolone
Antiepileptic drugs	Phenytoin, phenobarbital, topiramate, oxcarbazepine, carbamazepine-10,11-epoxide, eslicarbazepine acetate
DPP-4 inhibitors	Saxagliptin, sitagliptin
Antihypertensive agent	Aliskiren, ambrisentan, talinolol, tolvaptan, epidril
Diagnostic dyes	Calcein acetoxymethyl ester, Hoechst 33342, rhodamine 123, BCECF-AM, calcein AM
Others	Endosulfan, leupeptin, methyl parathion, paraquat, pepstatin A, transflupentixol, ivermectin, abamectin, emetine, reserpine, aristolochic acid, loperamide, dabigatran etexilate, ranolazine, digoxin, lovastatin, simvastatin, colchicines, domperidone, ondansetron, curcuminoids, flavonoids

Table 2.3 Selected examples of classical inhibitors of P-GP

First generation	Second generation	Third generation
Verapamil	(R)-verapamil	Tariquidar (XR9576)
Cyclosporin A	Valspodar (PSC-833)	Zosuquidar (LY335979)
Vincristine	Dexniguldipine	Laniquidar (R101933)
Reserpine	Elacridar (GF120918)	ONT-093 (OC144-093)
Quinidine	Biricodar (VX-710)	Mitotane (NSC-38721)
Tamoxifen	Dofequidar	Annamycin
Trifluoperazine		

that some drugs used clinically, including amiodarone, azithromycin, captopril, carvedilol, clarithromycin, conivaptan, cyclosporin A, diltiazem, dronedarone, erythromycin, felodipine, itraconazole, ketoconazole, lapatinib, lopinavir, indinavir, quercetin, quinidine, ranolazine, ritonavir, ticagrelor, telaprevir, and verapamil, are often strong inhibitors of P-GP. Coadministration of these drugs may elevate plasma exposure of other drugs (P-GP substrates) in plasma or tissues and increase their efficacy and toxicity.

Second-generation inhibitors including valspodar (PSC-833), dexniguldipine, elacridar (GF120918), biricodar, and dofequidar are compounds without therapeutic use. They have a higher affinity for P-GP than the first-generation compounds. Valspodar is a cyclosporin A analogue, but it does not have the immunosuppressive effect of cyclosporin A and can be given at quite high dosages to patients. Valspodar is a high-affinity but slowly transported substrate of P-GP. Valspodar is an efficient P-GP inhibitor; it is also an inhibitor of CYP3A4. Consequently, when administered to patients, in addition to inhibiting P-GP, valspodar may have additional effects on the clearance of drug substrates that are metabolized by CYP3A4. Many cytotoxic anticancer drugs such as etoposide and doxorubicin are both P-GP and CYP3A4 substrates. Therefore, coadministration with valspodar can enhance the toxic effects of these drugs, needing a dose reduction for safe treatment of the patient (Schinkel and Jonker 2012). Elacridar is a highly effective P-GP and BCRP inhibitor. In mice, elacridar treatment improved the response of implanted tumors to chemotherapy, via inhibiting P-GP and BCRP transporters (Schinkel and Jonker 2012).

The third-generation inhibitors are compounds that are designed using combinatorial chemistry and subsequent structure-activity relationship studies. These compounds often show high specificity for P-GP and low toxicity. They have a potency of about tenfold more than the earlier generations of inhibitors on P-GP function, without affecting CYP3A4 activity. LY335979 is a third-generation inhibitor, showing highly effective inhibition on P-GP function. It was reported to improve chemotherapy response in mice with transplanted tumors expressing P-GP without significantly altering plasma clearance of doxorubicin or etoposide following intraperitoneal administration (Dantzig et al. 1996). In line with the absence of pronounced plasma pharmacokinetic interactions, LY335979 is an extremely potent modulator of P-GP and not MRP1 or MRP2. The compound has a significantly lower affinity for CYP3A than for P-GP (Dantzig et al. 1999). XR9576 and OC144-093 appear to be very promising P-GP inhibitors as well. They can be given both orally and intravenously to improve the chemotherapy response of transplanted tumors expressing P-GP in mice, and they do not affect the plasma pharmacokinetics of intravenously administered paclitaxel (Mistry et al. 2001; Newman et al. 2000). A clinical trial showed that coadministration of tariquidar (XR9576) increased accumulation of docetaxel and vinorelbine in tumor by 22%, accompanied by decreases in clearance of two drugs (Fox et al. 2015). Further study showed that tariquidar is a potent inhibitor but not substrate of both human and mouse P-GP (Weidner et al. 2016).

Many inhibitors of P-GP including the third-generation compounds have been tested in clinical trials to assess their pharmacological potential, but it is unfortunate that most of them have failed (Binkhathlan and Lavasanifar 2013) due to following

issues: (1) non-specific toxicity; (2) high variability in the response rate associated with P-GP inhibitors, which is related to levels of P-GP expression and the co-expression of other ABC transporters; (3) interaction between the P-GP inhibitor and the substrates, leading to an increase in drug toxicity; and (4) the increase in the toxicity of a coadministered drug in healthy tissues by inhibiting the basal activity of P-GP. Therefore, there is an urgent need for identifying new, more effective, and nontoxic P-GP inhibitors without drug-drug interaction.

2.2.3 Physiological and Pharmacological Functions of P-GP

P-GP is mainly present in the apical membrane of epithelial cells in the body. As a consequence, P-GP substrates are transported from the basolateral to the apical side of the epithelium, which dramatically affects pharmacokinetic behaviors of substrate drugs, further altering their efficacy and toxicity.

2.2.3.1 Function of P-GP at Blood-Brain Barrier

Endothelial cells of the brain capillaries are closely linked to each other via tight junctions, forming blood-brain barrier (BBB). Function of BBB is to limit entrance of drugs to the brain, protecting the brain from toxic effects of exogenous compounds. P-GP is highly expressed in the luminal membrane of the brain microvessel endothelial cells. Most P-GP substrates are hydrophobic; theoretically they easily enter the brain across the endothelial cell membranes at a reasonable rate. However, P-GP at BBB pumps these drugs entering the endothelial cells back into blood, leading to dramatic decreases in the net penetration of substrate drugs from the blood into the brain. Contributions of P-GP to BBB have been verified by series of experiments. *Abcb1a/1b*$^{-/-}$ mouse studies have demonstrated that absences of P-GP at BBB could increase the brain penetration of P-GP substrates up to 10- to 100-fold (Geyer et al. 2009; Schinkel and Jonker 2012), in turn, increasing the clinical applicability and toxicity of compounds. Similarly, coadministration of P-GP inhibitors might increase brain distribution of P-GP substrates and potentiate central nervous system (CNS) activity of P-GP substrates (Liu et al. 2002; Liu et al. 2003). Some diseases may alter expression and function of P-GP at BBB, leading to alterations in CNS activity or toxicity of drugs (Jing et al. 2010; Liu et al. 2007; Fan and Liu 2018; Liu and Liu 2014).

2.2.3.2 Function of P-GP at Fetal-Maternal Barrier Function

P-GP is also located in apical membrane of the placental syncytiotrophoblasts. The syncytiotrophoblasts form the functional barrier between the maternal and fetal blood circulations and are essential for nutrient and waste product exchange but also for

protection of the fetal circulation. The function of P-GP in the placenta appears to be analogous to BBB, which protects the highly sensitive developing fetus from toxins and drugs present in the maternal circulation. The deduction has been directly demonstrated using *Abcb1a/1b*$^{-/-}$ mice on P-GP substrates (digoxin, saquinavir, and paclitaxel). To be compared with wild-type mice, fetal penetration of digoxin, saquinavir, and paclitaxel to pregnant dams of *Abcb1a/1b*$^{-/-}$ mice is 2.4-, 7-, and 16-fold of wild-type mice, respectively (Smit et al. 1999). For most therapeutic purposes, low penetration of drugs into the fetus is of course highly desirable. However, in some case, placental P-GP expression may become therapeutic obstacle. For example, highly active antiretroviral therapy containing HIV protease inhibitors is used to reduce the rate of mother-to-child HIV transmission (Capparelli et al. 2005), but most of HIV protease inhibitors are P-GP substrates. They cannot easily cross the placenta in an appreciable amount and cannot attend the desired drug concentration in utero during the whole dosing interval (Marzolini et al. 2002), inferring that coadministration of P-GP inhibitors may be a useful method to increase maternal fetal penetration of antivirotics and improve pharmacotherapy of the unborn child. Similarly, for the treatment of fetal tachycardia, it has been suggested that pharmacological inhibition of P-GP would be beneficial to enhance digoxin availability to the fetus, while minimizing drug exposure of the mother (Ito et al. 2001a).

2.2.3.3 Function of P-GP at Hepatobiliary Excretion

P-GP is very abundant in the bile canalicular membrane of hepatocytes, whose function is to extrude substrate drugs and other compounds from the liver hepatocyte into the bile. As many compounds can readily enter the hepatocytes from blood compartment, then they are excreted into bile via drug efflux transporters including P-GP. *Abcb1a/1b*$^{-/-}$ mouse studies directly have demonstrated roles of P-GP in biliary excretion of some drugs. The biliary excretion of vincristine is mediated by P-GP. In rats, coadministration of valspodar (0.1 mg/kg) was sufficient to significantly reduce the biliary excretion clearance of digoxin from 3.0 ml/min/kg to 0.5 ml/min/kg, but 3 mg/kg valspodar was needed to significantly reduce the biliary excretion clearance of vincristine from 36 ml/min/kg to 9 ml/min/kg (Song et al. 1999). Similarly, the biliary secretion of doxorubicin (2.4% of dose) in *Abcb1a*$^{-/-}$ mice was significantly lower than that (13.3% of dose) in wild-type mice (van Asperen et al. 2000). In human, coadministration of quinidine and quinine also decreased biliary clearance of digoxin from control value 134 mL/min to 87 mL/min and from 95 mL/min to 55 mL/min (Hedman et al. 1990), respectively.

2.2.3.4 Function of P-GP in Restricting Oral Bioavailability of Drugs

P-GP is abundant in apical side of the intestinal epithelium; one potential function is to limit entrance of some compounds or toxins from the intestinal lumen into the bloodstream. Many drugs are P-GP substrates, indicating that expression of

Table 2.4 Apparent values of Michaelis-Menten constant (K_m) for P-GP substrates from basolateral side to apical side

Compounds	Samples	Apparent K_m (μM)	References
Cyclosporin A	Caco-2	3.8	Lin and Yamazaki (2003)
Digoxin	Caco-2	58	Lin and Yamazaki (2003)
	Human colon	59	Lin and Yamazaki (2003)
Etoposide	Caco-2	213	Lin and Yamazaki (2003)
Indinavir	Caco-2	140	Lin and Yamazaki (2003)
Paclitaxel	Caco-2	16.5	Walle and Walle (1998)
Verapamil	Caco-2	1.01	Tachibana et al. (2010)
Vinblastine	Caco-2	80.7	Tachibana et al. (2010)
Quinidine	Caco-2	1.69	Tachibana et al. (2010)

intestinal P-GP contributes to low bioavailability of many orally administered drugs. For example, oral bioavailabilities of paclitaxel in wild-type and *Abcb1a*$^{-/-}$ mice are 11% and 35%, respectively. The cumulative fecal excretions following intravenous and oral administration to wild-type mice are 40% and 87% of dose, respectively. But, the cumulative fecal excretions in *Abcb1a*$^{-/-}$ mice are less than 3% of dose (Sparreboom et al. 1997). Elacridar markedly increased the oral bioavailability of paclitaxel in wild-type mice from 8.5 to 40.2% (Bardelmeijer et al. 2000). Coadministration of elacridar also significantly increased the systemic exposure to oral paclitaxel in cancer patients (Malingré et al. 2001). Oral drug administration is highly preferred because it is cheap, relatively safe, and patient-friendly. Therefore, it is of interest for pharmaceutical companies at an early stage to check whether candidate is a good P-GP substrate.

The functional activity of P-GP is saturable, which may be illustrated using Michaelis-Menten equation. Michaelis-Menten constants (K_m values) for vinblastine, vinblastine, digoxin, and cyclosporin were estimated to be 26, 18, 58, and 3.8 μM using Caco-2 cells (Table 2.4). The K_m values of digoxin in human colon were measured to be 59 μmol/L using the Ussing chamber technique, which is almost identical to that 58 μM from Caco-2 cells. Above results suggest that the activity of intestinal P-GP may be saturated when drug concentrations in the intestinal lumen exceed their K_m values. The saturable P-GP efflux may, at least in part, explain the following phenomena. (1) Intestinal absorption of some drugs is increased along with dose. For example, in the healthy volunteers, the dose-normalized area under the curve (*AUC*) of (*S*)-(−)-talinolol was 18 μg. h/L following oral 12.5 mg of talinolol but increased to 36 μg h/L following oral 200 mg of talinolol. Similar results were observed for (*R*)-(+)-talinolol (Lin and Yamazaki 2003; Wetterich et al. 1996). Saturable efflux mediated by P-GP was also demonstrated for cyclosporin A in rats (Lindberg-Freijs and Karlsson 1994). The bioavailability data following oral doses of 3.1, 6.8, and 12.9 mg/kg were measured to be 45%, 67%, and 76%, respectively. (2) Absorption of drug at low doses is greatly limited by P-GP. Absorption of digoxin is a good example. Digoxin is a well-known P-GP substrate and undergoes minimal metabolism. In clinic, oral dose is only

0.5–1 mg. At the doses, the concentration of digoxin in intestinal lumen is estimated to be less than 10 μM, which is less than its K_m (58 μM). The low bioavailability and high variety in absorption of digoxin can be mainly attributed to intestinal P-GP. However, the oral dose of most drugs is high (>50 mg), concentrations of these drugs in the intestinal lumen can often reach the mmol/L concentration range, which excesses their K_m values. Thus, P-GP activity can readily be saturated, and its role in drug absorption becomes less significant. An example is indinavir, a P-GP substrate, whose clinical dose is high to 800 mg. At this dose, the indinavir concentration in the intestinal lumen is higher than its K_m value (140 μM). Thus, the role of P-GP in intestinal absorption of indinavir is negligible, which can explain why indinavir has a reasonably good bioavailability (>60%) in patients, although it is a good P-GP substrate. (3) There are some exceptions that intestinal P-GP still plays a significant role in absorption for some drugs, even when they are given at high doses. For example, the clinical oral doses of cyclosporine A and paclitaxel are 200–700 mg and 100–200 mg, respectively. Clinical studies clearly indicate that their oral absorption is greatly limited by intestinal P-GP. This can be explained by the fact that both cyclosporin A and paclitaxel have very poor water solubility and slow dissolution rate, resulting in low drug concentration in the intestinal lumen.

It should be noted that expression of intestinal P-GP and its contributions to intestinal absorption are region-dependent. In human, relative expression of intestinal P-GP progressively increases from proximal to distal regions. Jejunal region shows the highest expression of P-GP (Mouly and Paine 2003). Similar phenomena are shown in the intestine of rats (Dahan et al. 2009; Dahan and Amidon 2009; Yu et al. 2010) and mice (Stephens et al. 2002). The P-GP levels in segment of the intestine were inversely related to the in vivo permeability of the drugs from the segments (Dahan and Amidon 2009). The influence of uneven distribution of P-GP in the intestine was also demonstrated in a clinical study with cyclosporin A (Fricker et al. 1996). Cyclosporin A (150 mg) was given to ten healthy volunteers via different parts of the gastrointestinal tract (stomach, jejunum, and colon). The oral *AUC* of cyclosporin is in the rank order stomach (2980 ng.h/mL) > jejunum (1570 ng.h/mL) > colon (61 ng.h/mL). C_{max} values show similar rank order. Oral *AUC* of cyclosporin A was negatively related to ABCB1 mRNA expression in corresponding segment. All these indicate that the permeability of P-GP substrate drugs across the gastrointestinal wall is dominated by P-GP in the distal small intestine, whereas efflux transport is insignificant in the proximal intestinal segments.

2.2.3.5 P-GP Function in the Kidney

In the kidney, P-GP is identified in the mesangium, proximal tubules, the thick limb of Henle's loop, and the collecting duct, whose function is considered to pump substrates from blood into urine. In dog kidney, the single-pass multiple indicator dilution method showed that coadministration of P-GP inhibitors cyclosporin A and quinidine both decreased urine recovery of [^{3}H]digoxin without affecting glomerular

extraction (De Lannoy et al. 1992). Similarly, in the isolated perfused rat kidney, renal tubular secretion of digoxin was inhibited by cyclosporin A (Okamura et al. 1993). In line with these findings, digoxin content in the kidney of *Abcb1a/1b*$^{-/-}$ mice was sharply higher than that of wild-type mice (Tsuruoka et al. 2001). Collective results infer role of renal P-GP in urinary excretion of digoxin. However, the cumulative urinary excretion of the digoxin in *Abcb1a*$^{-/-}$ mice was also reported to be increased compared with wild-type mice (Mayer et al. 1996). This dissimilarity may be due to the activity of other transporters, e.g., Abcb1b P-GP, for which the mRNA levels were reported to be increased in the mice with disrupted *Abcb1*a gene (Schinkel et al. 1994).

Cyclosporin A has proved to be a successful agent in the prevention and treatment of allograft rejection and in autoimmune diseases. However, severe nephrotoxicity has been associated with this agent. Cyclosporin A is a typical P-GP substrate and variations in expression and/or function of P-GP can lead to accumulation of cyclosporin A within the tubular cells. In support of this hypothesis, immunohistological studies (Del Moral et al. 1997) showed an inverse relationship between cyclosporin A deposits in renal tissue and the level of kidney P-GP expression in animal models. Clinical trials demonstrated that patients with cyclosporin A nephrotoxicity exhibited less expression of P-GP in the arterial endothelial cells and proximal tubules (Koziolek et al. 2001). In normal human renal epithelial cells, P-GP inhibitors verapamil, valspodar, and quinine enhanced cyclosporin A cytotoxicity (Anglicheau et al. 2006). These results suggest that the renal P-GP response may be defective in patients susceptible to cyclosporine A-related nephrotoxicity, leading to retention of excess amounts of cyclosporin A in the cells.

Renal P-GP is inducible. Cyclosporin A treatment also induces P-GP expression in parenchymal cells of kidney transplants with acute tubular necrosis, acute or chronic transplant rejection. The increased expression of P-GP in infiltrating leukocytes was also correlated with the severity of allograft rejection, suggesting that decrease in the immunosuppressive efficacy of cyclosporin A may partly be attributed to upregulation of P-GP expression (Koziolek et al. 2001). The proximal tubule of the kidney is a major target of chronic cadmium-induced toxicity. In vivo and in vitro studies showed that chronic exposure of cadmium upregulated P-GP expression in kidney proximal tubule cells. The overexpression of P-GP can protect proximal tubule cells against cadmium-mediated apoptosis, which was reversed by valspodar (Thévenod et al. 2000).

2.2.3.6 P-GP Function in Immune System

P-GP is also expressed in a variety of immune cells like monocytes, antigen-presenting dendritic cells, T cells, and B cells (Frank et al. 2001) and involves the efflux of inflammatory molecules such as steroids, prostaglandins, and cytokines (Barnes et al. 1996; Drach et al. 1996; Ernest and Bello-Reuss 1999; Frank et al. 2001; Meijer et al. 1998; Raggers et al. 2001). Clinical trial demonstrated a negative relationship between clinical sensitivity to glucocorticoid therapy and P-GP function

of $CD4^+$ cells in systemic lupus erythematosus patients (Henmi et al. 2008). In normal peripheral T lymphocytes, both P-GP inhibitors (verapamil and tamoxifen) and P-GP-specific monoclonal antibody inhibited releases of cytokines (IL-2, IL-4, and IFN-y) stimulated by phytohemagglutinin. Transport of IL-2 across HCT-8 monolayers was partially inhibited by verapamil (Drach et al. 1996). In *Abcb1a/1b*$^{-/-}$ mice, the function of the dendritic cells was severely impaired, leading to significant decreases in cell maturation and T-cell stimulatory capacity. Adding exogenous cytokines may restore defect in dendritic cell function. *Abcb1a/1b*$^{-/-}$ mice developed the decreased clinical signs of experimental autoimmune encephalomyelitis, which was associated with impaired T-cell responses and T-cell-specific brain inflammation (Kooij et al. 2009, 2010). In vitro studies also demonstrated that differentiation and maturation of dendritic cell were dependent on P-GP. The downregulation of P-GP by venlafaxine inhibited the differentiation of dendritic cell and cytokine production, such as IL-1, IL-10, and IL-12 during dendritic cell maturation. In addition, P-GP of dendritic cell is required for dendritic cell-mediated T-cell polarization and proliferation (Lee et al. 2011).

The roles of P-GP in development of inflammatory bowel disease have been demonstrated. Allelic variants of *ABCB1* gene are linked to both Crohn's disease and ulcerative colitis, the two main forms of human inflammatory bowel disease (Brinar et al. 2013). *Abcb1a*$^{-/-}$ mice are served as animal model of spontaneous colitis (Masunaga et al. 2007; Nones et al. 2009a, b; Panwala et al. 1998). However, incidence of colitis in *Abcb1a*$^{-/-}$ mice treated with antibiotics was greatly reduced, suggesting that the presence of the gut flora is necessary for the development of colitis (Panwala et al. 1998). Moreover, upregulation of cytokines and chemokines occurs in the large intestine and mesenteric lymph node cells from *Abcb1a*$^{-/-}$ mice, including interferon-γ (IFN-γ), interleukin 6 (IL-6), interleukin 11β (IL-1β), tumor necrosis factor-α (TNF-α), chemokine ligand 2 (CCL2), macrophage inhibitory protein-1 α (MIP1α), and chemokine ligand 5 (CCL5) (Masunaga et al. 2007). The lack of P-GP in intestinal cells may lead to changes in the cecal microbiota. The altered microbiota along with the genetic defect can contribute to the development of intestinal inflammation in *Abcb1a*$^{-/-}$ mice (Nones et al. 2009b). Data from human monocyte THP-1 cell lines showed that *L. monocytogenes* infection upregulated expression of MDR1 mRNA, which directly correlated with IFN-β levels elicited by listerial strains. Verapamil treatment reduced IFN-β levels in infected THP-1 cells. Similarly, silencing of P-GP transcription led to a reduced type I interferon response upon *L. monocytogenes* infection. These results indicate that P-GP is involved in stimulation of the IFN-β response (Sigal et al. 2015).

2.2.4 Pharmacological Significance of P-GP Inhibition

Oral bioavailability is an important parameter for the practical use of many drugs, and direct inhibition of intestinal P-GP may improve oral bioavailability of P-GP substrates. The first directed attempts to improve oral bioavailability of paclitaxel

were made in both mice and humans. P-GP inhibitors valspodar, elacridar, and even cyclosporin A can dramatically increase oral availability (Schinkel and Jonker 2012). Clinically, P-GP inhibitors are being tried to whether such treatment protocols have clear therapeutic benefit in cancer treatment.

A partly related application would concern the chemotherapy treatment of tumors (e.g., gliomas) that may be positioned behind BBB and therefore poorly accessible to most anticancer drugs. For other CNS diseases, it may likewise be desirable to improve the brain parenchyme penetration of drugs. P-GP is involved in transport of nimodipine across BBB. We once reported that coadministration of erythromycin and cyclosporin A significantly enhanced brain exposure of nimodipine following intravenous administration (2 mg/kg). The estimated $T_{1/2}$ of nimodipine in the brain of rats co-treated with erythromycin (75.0 min) and cyclosporin A (79.0 min) was larger than that (44.2 min) in rats treated with nimodipine alone (Liu et al. 2003). In consistence, cyclosporin A may also enhance the protection of nimodipine against brain damage in rats and mice (Liu et al. 2002). In some cases it may be desirable to increase the penetration of drugs into the fetus, although in general one would prefer to minimize fetal drug penetration to prevent toxic effects. For efficacious anti-HIV drugs, it may be useful to increase fetal drug penetration in the period shortly before delivery to minimize the chance that there will be mother-to-child transmission of the virus during birth. Mouse study showed that both valspodar and elacridar could substantially increase the fetal penetration of saquinavir (Huisman et al. 2001, 2003). This procedure in humans should be carefully investigated to assess the risk of unexpected toxicities for the unborn child.

In addition, it should be noted that unplanned P-GP inhibition, owing to coadministration of several drugs, sometimes results in unwanted toxicity effects. Digoxin is a good example. Digoxin, a narrow therapeutic window, has been used for centuries to treat heart failure, although life-threatening ventricular tachyarrhythmias and severe bradyarrhythmias often occur. Dronedarone, an antiarrhythmic drug, is also a strong P-GP inhibitor. It was reported in a case of digoxin toxicity in a patient taking concomitant dronedarone (Vallakati et al. 2013). A clinical trial in a 15-year, population-based, nested case-control study also demonstrated the association between hospitalization for digoxin toxicity and exposure to individual macrolide antibiotics. Clarithromycin showed the highest risk of digoxin toxicity, whose adjusted odds ratio (OR) and 95% confidence interval (CI) were 14.8 and 7.9–27.9, respectively. Erythromycin (adjusted OR 3.7; CI 1.7–7.9) and azithromycin (adjusted OR 3.7; CI 1.1–12.5) were associated with much lower risk (Gomes et al. 2009). Pharmacokinetic study showed that coadministration of clarithromycin (250 mg) increased plasma exposure of digoxin by 1.7-fold, which was partly due to increasing oral bioavailability and reducing non-glomerular renal clearance of digoxin (Rengelshausen et al. 2003). In pig, it was reported that coadministration of quercetin (50 mg/kg) unexpectedly resulted in sudden death of two among three pigs within 30 min after oral administration of digoxin (0.02 mg/kg), another pig was also intoxicated, but three pigs receiving digoxin alone showed no toxic effect. Pharmacokinetics demonstrated that the coadministration of 40 mg/kg quercetin significantly elevated the C_{max} and AUC^{0-24} of digoxin by 413% and 170% (Wang et al. 2004), respectively.

Another example is colchicine, widely used for familial Mediterranean fever and gout. Colchicine is typical P-GP substrate and primarily eliminated through biliary excretion. It was reported that a 76-year-old man with familial Mediterranean fever following coadministration of clarithromycin showed dehydration, pancytopenia, metabolic acidosis, and increased lipase level necessitated hospitalization (Rollot et al. 2004). Indeed, subsequent reports demonstrated that clarithromycin and cyclosporine A increased colchicine-induced rhabdomyolysis (Bouquié et al. 2011; Cohen et al. 2015).

2.2.5 ABCB1 *Polymorphisms and P-GP Expression/ Function*

The *ABCB1* gene encoding P-GP is highly polymorphic. Till date, 66 coding single-nucleotide polymorphisms (SNPs) in *ABCB1* gene have been identified. Out of these, 22 are synonymous and 44 nonsynonymous. Three SNPs (*c.2677G > T/A*, *c.1236C > T*, and *c.3435C > T*) in the exonic region of MDR1 frequently occur. The *c.2677G > T/A* polymorphism is a triallelic variant, and it is found in the wild-type sequence with G at nucleotide 2677 and in the variant sequence with A or T. It contains a nonsynonymous amino acid change from Ala at codon 893 to Ser/Thr (Ala893Ser/Thr). The other two SNPs *c.3435C > T* (p.Ile1145Ile) and *c.1236C > T* (p.Gly412Gly) are synonymous SNPs. The *c.3435C > T* is present in exon 26 of the *ABCB1* gene and does not alter the amino acid isoleucine at codon 1145. *c.1236C > T* is present in exon 12 of the *ABCB1* gene. Its amino acid glycine at codon 412 is not altered.

An important characteristic of these SNPs is that large interethnic difference in their allele frequency varies. Frequencies of *3435CC* genotype in 172 West Africans, 41 African Americans, 537 whites, and 50 Japanese were identified to be 83%, 61%, 26%, and 34% (Schaeffeler et al. 2001), respectively. On the contrast, West Africans showed the lowest frequency of *3435TT* genotype. The order rank of *3435TT* genotype was West Africans (1.7%) < African Americans (4.9%) < Japanese (20%) < Caucasians (26.4%). In accordance, allelic frequencies of c.*1236 C > T*, *c.2677 G > A*, *c.2677 G > T*, and *c.3435 C > T* in 154 Japanese were identified to be 65.6%, 16.6%, 40.6%, and 40.6%, respectively. Frequencies of the SNPs in 100 Caucasians were 45.9%, 3.6%, 46.4%, and 56.6%, respectively. Frequency of *c.2677G > A* in Japanese was about fivefold more frequent than that in Caucasians (Komoto et al. 2006). Large interethnic difference in *ABCB1* SNPs seems to explain to interethnic differences in disposition of P-GP substrate drugs.

Much interest has been focused on *c.3435C > T*. The association of *c.3435C > T* with P-GP expression in the duodenum of healthy Caucasians was first reported by Hoffmeyer et al. (2000). Their reports showed that the carriers with *3435CC* genotypes had on average more than twofold high intestinal P-GP expression levels compared to the *3435TT* genotype. Subjects with *3435CT* had on average

intermediate P-GP expression. In consistence with P-GP expression, the lowest plasma exposure (*AUC* and C_{max}) of digoxin following oral dose was found in CC genotypes. P-GP expression was also associated with *c.3435C > T* genotype (i.e., CC > CT > TT) in placentas of 100 Japanese women although no significance was obtained (Tanabe et al. 2001). In human kidney, subjects with the 3435TT genotype had on average a significantly (1.5-fold) lower P-GP expression compared to the CC genotypes (Siegsmund et al. 2002). Data from CD56+ natural killer cells demonstrated that cellar rhodamine fluorescence was *3435CC < 3435CT < 3435TT*, which was negatively related to *ABCB1* mRNA expression. The lowest *ABCB1* mRNA expression was found in cells of 3435TT population, although no significance was obtained (Hitzl et al. 2001).

Several studies have addressed the association of *ABCB1* genotypes with disposition of P-GP substrates in humans. Johne et al. (2002) reported that plasma concentration-time curve from time zero to 4 h (AUC^{0-4}) and C_{max} values of digoxin at steady state in Caucasians with the 3435TT genotypes were higher than those with 3435CC genotypes. Haplotype analysis demonstrated that subjects with the 2677G/3435T genotypes exhibited higher AUC^{0-4} (5.7 ± 0.9 μg.h/L) values than that in noncarriers (4.8 ± 0.9 μg.h/L). On the contrast, subjects with *2677G/3435C* genotypes had lower AUC^{0-4} values (4.7 ± 0.9 μg.h/L) than noncarriers (5.6 ± 0.9 μg.h/L). A report showed a significant relationship between *3435CT* SNP and digoxin AUCs. The *3435TT* subjects had 20% higher digoxin plasma concentrations than *3435CT* and *3435CC* subjects and a trend for higher 48 h digoxin urinary recoveries (*3435TT > 3435CT > 3435CC*) (Verstuyft et al. 2003). In agreement with these, Niemeijer et al. (2015) reported that in digoxin users, homozygous T allele carriers of *1236CT*, *2677GT*, and *3435CT* were associated with an increased risk of sudden cardiac death compared with digoxin users with none or one T allele.

However, there exist conflicting reports. For example, several reports showed that *ABCB1* SNPs did not influence disposition of digoxin (Gerloff et al. 2002; Kurzawski et al. 2007), fexofenadine (Drescher et al. 2002), and cyclosporin A (Anglicheau et al. 2004). In vitro studies on *Xenopus laevis* oocytes expressing triple SNP variant of P-GP (1236C > T, 2677G > T, and 3435C > T) showed that triple SNP variant of P-GP did not molecularly transport digoxin or imatinib (Dickens et al. 2013). On the contrast, Sakaeda et al. (2001) reported higher serum concentrations of digoxin after a single administration in healthy Japanese subjects with *3435CC* compared with that of *3435 CT* or *TT*. Asano et al. (2003) reported that in renal transplant patients, *3435TT* genotype exhibited a significantly lower incidence of non-traumatic osteonecrosis of the femoral head and higher concentration/dose ratio of tacrolimus than *3435CC* genotype. In addition, lower plasma concentrations of efavirenz and nelfinavir were also found in subjects with *3435 TT* genotype (Fellay et al. 2002). These data indicate that P-GP activity in patients with *3435TT* genotype is increased, not decreased.

These conflicting results may be due to the following confounding factor:

1. Linkage disequilibrium between the *c.3435C > T* SNP and the other genetic variants. The *c.3435 C > T* SNP is a wobble SNP; it may not be the sole SNP

affecting P-GP expression but rather may be functioning in concert with other putative SNPs at other loci in the *ABCB1* gene.
2. Different patient population as well as diversity of substrate drugs.
3. The contribution of *ABCB1* SNPs to drug disposition is rather modest. Disposition of most P-GP substrates is also determined by other factors such as metabolism (e.g., nelfinavir or cyclosporin metabolism via CYP3A4) or transport (e.g., fexofenadine or digoxin uptake via OATPs).
4. Drug disposition may be affected by some exogenous factors (e.g., diet and drugs).
5. Too small sample sizes.

Linkage disequilibrium between *c.3435C > T* SNP and other SNPs (such as *c.1236C > T* and *c.2677G>T/A*) often occurs. Linkage disequilibrium among *ABCB1* SNPs also exhibits large interethnic differences (Fung and Gottesman 2009). For example, in French populations, frequencies of *3435/1236/2677 CCG* and TTT genotypes were reported to be 44.00% and 35.30%, respectively. In Chinese populations, frequencies of CCG and TTT were 16.80% and 35.70%, respectively. But in a Beninese, frequencies of *3435/1236/2677CCG* and TTT were 79.30% and 4.50%, respectively. Frequency of *3435/1236/2677 TTT* genotypes in French populations was eightfold of that in Beninese. Analysis of the haplotype structure across the entire *ABCB1* gene in different populations aids to identify associations between *ABCB1* SNPs and P-GP expression/function. In an Asian population (Chinese, Malays, and Indians) (Tang et al. 2002), three SNPs (*c.1236C > T*, *c.2677G > T/A*, and *c.3435C > T*) were all present in high frequency, whose haplotype structure exhibited distinct differences between the groups. Malays exhibited fewer haplotypes than Chinese and Indians did. Three major haplotypes were observed in the Malays and Chinese; of these, two were observed in the Indians. Strong linkage disequilibrium was detected between the three SNPs in all three ethnic groups. Singh et al. (2011) investigated effect of *ABCB1* SNPs on individualizing cyclosporin A and tacrolimus dosage and subsequently the allograft outcome in renal transplant recipients. The results showed that both GG genotype patients at *c.2677G > T* and CC genotype patients at *c.3435C > T* were associated with lower dose-adjusted levels of cyclosporin A and tacrolimus at 1 month, 3 months, and 6 months posttransplantation. Wild-type patients at *c.1236C > T* and *c.2677G > T* in cyclosporin A and *c.2677G > T* and *c.3435C > T* in tacrolimus therapy possessed lower meantime to allograft rejection. But, no influence of *ABCB1* haplotypes on cyclosporin A/tacrolimus dose-adjusted levels was observed. Wild-type patients at *c.2677G > T* and *c.3435C > T* were associated with lower dose-adjusted levels and thereby were at increased risk of allograft rejection. Effects of *c.2677G > T/A* and *c.1236C > T* on tacrolimus concentrations were also demonstrated in a renal transplant patients (Mendes et al. 2009). Patients who had encoded the *c.1236C > T* displayed 44.4% higher tacrolimus concentrations compared with wild-type individuals. Individuals carrying the *c.2677G > T/A* mutation showed 44.7% higher values than wild-type individuals (Mendes et al. 2009). However, Spanish TT carrier patients on C3435T, G2677 T, and C1236T SNPs (P-GP-low

pumpers) were reported to show lower P-GP activity than noncarriers based on peripheral blood mononuclear cells using the rhodamine 123 efflux assay, a negative correlation between cyclosporine *AUC* or C_{min} and P-GP activity at 1 months. This negative correlation did not occur in patients treated with tacrolimus nor sirolimus (Llaudó et al. 2013).

A study in Asian renal transplant recipients (Loh et al. 2008) demonstrated that the median concentration/dose ratios of tacrolimus in patient with *3435CC* genotype was significantly higher than that in *3435CT* genotypes or *3435TT* genotypes, whose order rank was CC > CT > TT. The findings were contrast to the hypothesis that wild-type allele carriers would have higher P-GP activity in their intestinal cells, which in turn would effectively pump substrate drug into the intestinal lumen, leading to low concentration. Further study showed that all *3435CC* carriers were also *CYP3A5∗3∗3*, which may partly explain high concentration/dose ratios of tacrolimus in patients with *3435CC*. However, concentration/dose ratios of cyclosporin A was not associated with *c.3435C > T*. Lee et al. (2015) evaluated the influence of *c.3435C > T* on the dose-adjusted trough (C_0/D) and peak (C_{max}/D) concentrations of cyclosporin A based on a literature search of four authoritative databases, 13 studies concerning 1293 kidney transplant recipients using meta-analysis. Their result showed a significant difference of C_0/D and C_{max}/D between *3435CC* and *3435TT* genotype carriers. Subgroup analysis by ethnicity demonstrated that C_0/D was lower in Asian CC than TT genotype carriers, but this difference did not occur in Caucasian. Moreover, significant variation of C_0/D was found at 1 week and 1–3 months after transplantation between CC and TT genotype carriers. The meta-analysis showed that patients with *3435CC* genotype will require a higher dose of cyclosporin A to achieve target therapeutic concentrations than *3435TT* carriers after kidney transplantation.

Effect of *ABCB1* SNPs on the incidence of nephrotoxicity and dosage requirement of tacrolimus was investigated in liver transplant pediatric patients (Hawwa et al. 2009). The estimated glomerular filtration rate (GFR) was used for indexing nephrotoxicity. Higher incidences of the *ABCB1* variant alleles were associated with renal dysfunction (about 30% reduction in GFR) at 6 months posttransplantation (*1236 T* allele, 63.3% versus 37.5% in controls; *2677 T* allele, 63.3% versus 35.9%; and *3435 T* allele, 60% versus 39.1%). Carriers of the *2677 T* allele also had a significant reduction in GFR at 12 months posttransplant. Haplotype analysis showed a significant association between T-T-T haplotypes and an increased incidence of nephrotoxicity at 6 months posttransplantation (52.9% versus 29.4%). Furthermore, *c.2677G > T* and *c.3435 C > T* SNPs and T-T-T haplotypes were significantly correlated with higher tacrolimus concentration/dose ratio.

Numerous clinical studies have demonstrated that *ABCB1* polymorphisms, especially the *c.3435C > T* mutation, are predictive factors in the onset of certain diseases. For example, a report demonstrated that the *3435TT* genotype was associated with occurrence of acute lymphoblastic leukemia (ALL) in 113 children with ALL and 175 healthy individuals. Analysis of factors influencing clinical outcome of the ALL patient cohort showed that *3435CC* genotype carriers had significantly lower event-free survival probability and overall survival probability. The results

infer that carriers of the *3435TT* genotype are more at risk of developing ALL than other individuals, whereas *3435CC* genotype carriers are supposed to have worse prognosis (Jamroziak et al. 2004). A report (Hattori et al. 2007) verified that *ABCB1* SNPs were associated with occurrence of ALL in 157 children with ALL and 96 healthy individuals. The frequencies of *2352GG* genotype and *3435TT* genotype were significantly higher in ALL patients than in healthy subjects. Patients aged 6 or older had the *2352 GG* genotype more frequently than the controls. The expressions of *ABCB1* mRNA were significantly higher in either G/G or G/A genotype of the c.*2352 G > A* than in A/A genotype.

2.3 Multidrug Resistance-Associated Proteins (MRPs/ABCCs)

2.3.1 Multidrug Resistance-Associated Protein 1 (MRP1/ABCC1)

2.3.1.1 General Properties and Distribution of MRP1

MRP1/Mrp1 (gene symbol *ABCC1* for human and *Abcc1* for animals, MW190 kDa) was originally identified as the mediator of acquired drug resistance in a small-cell lung cancer cell line selected by repeated exposure to doxorubicin. In mammalian cells, the MRP1 protein is both N-glycosylated and phosphorylated. Human MRP1 contains 1531 amino acids with 2 NBDs and 17 TMs in 3 TMDs (TMD0, TMD1, and TMD2). MRP1 mRNA and/or protein is frequently detected in tumor samples from patients. MRP1 is currently considered to be the most clinically relevant of the MRPs with respect to drug resistance in cancer, a major obstacle to successful chemotherapy. In addition to tumor cells, MRP1 is also expressed in most tissues throughout the body with relatively high levels found in the lung, testis, kidneys, skeletal muscle, brain, and peripheral blood mononuclear cells, while relatively low levels are found in the liver. MRP1 localizes to the basolateral surface of epithelia and the apical surface of brain capillaries, generally resulting in the efflux of MRP1 substrates into the blood, inferring that MRP1 contributes to drug and xenobiotic disposition in normal cells and important roles in tissue defense.

Human MRP1 and the murine ortholog of Mrp1 share 88% amino acid identity. Nucleotide and protein sequences of MRP1/Mrp1 from several other animal species including rat, canine, bovine, and monkey share 88%, 92%, 91%, and 98% amino acid identity, respectively, with human MRP1 (Leslie et al. 2005). Despite the high level of identity between human MRP1 and other species MRP1/Mrp1, some remarkable differences in substrate specificity exist (Leslie et al. 2005; Nunoya et al. 2003). For example, Mrp1 orthologs of mice and rats confer negligible levels of resistance to three anthracyclines (doxorubicin, daunorubicin, and epirubicin), while human MRP1 shows strong resistance to the three anthracyclines. On other hand, MRP1/Mrp1 orthologs of rats, mice, and human

showed similar resistance to vincristine. The transport of estradiol-17β-D-glucuronide ($E_2$17βG) by mouse Mrp1 was similar to that of rat Mrp1 but was less than 10% that of human MRP1 (Nunoya et al. 2003).

2.3.1.2 Substrates and Inhibitors of MRP1

It is well established that MRP1 can confer resistance to many widely used antineoplastic drugs including etoposide, teniposide, vincristine, vinblastine, doxorubicin, daunorubicin, epirubicin, idarubicin, topotecan, irinotecan (SN-38), and mitoxantrone as well as inhibitors of some signal transduction pathways (e.g., tyrosine kinase inhibitors). Antivirals (saquinavir and ritonavir), some fluorescent probes (calcein, Fluo-3, and BCECF), toxicants (aflatoxin B1, methoxychlor, fenitrothion, chlorpropham), and inorganic heavy metal oxyanions (arsenite and trivalent antimony) are also substrates of MRP1. Generally MRP1 mainly transports structurally diverse amphipathic organic anions, most of which are conjugated with glutathione (GSH), glucuronide, or sulfate. These conjugates are typically products of phase II drug metabolism. Thus, MRP1 (or MRP2)-mediated transport is also the so-called phase III elimination pathways of drug. The most conjugates are nontoxic; however, there are exceptions. For example, conjugates of *N*-methyl-α-methyldopamine and 3,4-dihydroxyamphetamine with GSH were identified to be strong ecstasy (Jones et al. 2005), whose efflux from the brain is mediated by MRP1 (or MRP2) (Slot et al. 2008).

MRP1 also transports endogenous organic anion conjugates with GSH, glucuronide, or sulfate. The best characterized endogenous substrates of MRP1 are cysteinyl leukotriene C4 (LTC4) and $E_2$17βG. Other signaling molecules such as prostaglandin A, 15-deoxy-ΔPGJ, sphingosine-1-phosphate, lysophosphatidylinositol, and 4-hydroxynonenal-glutathione conjugate are also endogenous substrates of MRP1. In addition, estrone sulfate, dehydroepiandrostenedione sulfate, bilirubin and its glucuronides, and glucuronide/sulfate-conjugated bile salts are transported by MRP1. MRP1 also mediates transport of GSH and glutathione disulfide (GSSG), although GSH possess low affinity to MRP1.

Interaction between MRP1 and its substrates is grouped into five categories.

1. MRP1 efflux many GSH conjugates (Fig. 2.3a), including LTC4 and most of GSH conjugates.
2. Efficient transport of MRP1 substrates requires the presence of GSH, but the transport does not involve the formation of GSH conjugates. For example, vincristine and mitoxantrone are only efficiently transported by MRP1 in the presence of GSH. On other hand, vincristine and mitoxantrone stimulate GSH transport, inferring a cotransport mechanism (Fig. 2.3b).
3. GSH markedly increases transport of MRP1 substrates, including several glucuronates (e.g., etoposide glucuronide), sulfate (e.g., estrone sulfate and dehydroepiandrosterone sulfate), and GSH conjugates (e.g., 4-nitroquinoline 1-oxide-SG). But these substrates do not stimulate GSH transport, indicating a GSH-stimulated mechanism (Fig. 2.3c).

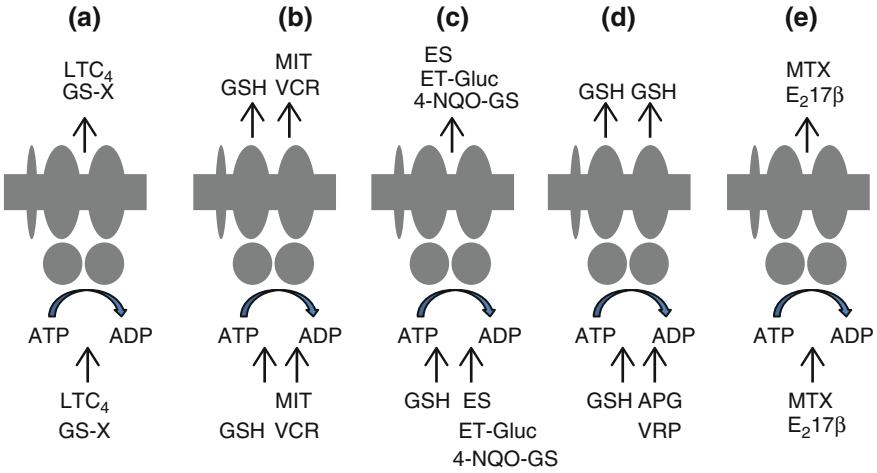

Fig. 2.3 Schematic representation of MRP1-mediated transport that involves GSH. (**a**) GS-conjugated transport, (**b**) GSH-drug cotransport, (**c**) GSH-stimulated conjugated transport, (**d**) drug-stimulated GSH transport, and (**e**) GSH-independent transport. *APG* apigenin, *ES* estrone sulfate, *ET-Gluc* etoposide glucuronide, *GS-X* GSH conjugate, *MIT* mitoxantrone, *MTX* methotrexate, *4-NQO-GS* 4-nitroquinoline 1-oxide-SG, *VCR* vincristine, *VRP* verapamil

4. GSH itself is a relatively poor substrate of MRP1, but some compounds such as verapamil and apigenin significantly enhance GSH transport by MRP1. There is no evidence that verapamil and apigenin are themselves transported by MRP1, inferring that GSH transport by MRP1 occurs both by a cotransport or cross stimulated mechanism and by a xenobiotic-stimulated mechanism (Fig. 2.3d).
5. MRP1-mediated transport of some organic anions, including GSSG, methotrexate, $E_2$17βG, and some other glucuronides, is independent of GSH (Fig. 2.3e).

Real mechanisms leading to substrate specificity differences are not fully understood, but several reports (Maeno et al. 2009, Ito et al. 2001b) have demonstrated roles of Lys332 in TM 6 and Trp1246 in TM17 in substrate selectivity of MRP1 transport. TM6-Lys332 mutant selectively eliminates LTC4 (and GSH) transport without affecting methotrexate. TM17-Trp1246 mutant no longer transports methotrexate and $E_2$17βG. Both mutants almost abolish GSH-stimulated estrone 3-sulfate transport. TM17-Trp1246, not TM6-Lys332, mutant abolishes GSH-independent estrone 3-sulfate transport. In wild-type MRP1, apigenin (10 μM) alone had no effect on $E_2$17βG uptake, whereas GSH (3 mM) alone decreased $E_2$17βG uptake by just 35%. Coadministration of apigenin and GSH decreased $E_2$17βG uptake by 70%. On the contrast, neither GSH or apigenin alone nor the combination of GSH and apigenin had any effect on $E_2$17βG uptake by the TM6-Lys332 mutant, inferring that GSH binding to the TM6-Lys332 mutant is severely impaired. Both two mutants decrease affinity of estrone 3-sulfate to MRP1, which are only 20% of wild-type MRP1. Unlike wild-type MRP1, *S*-MeGSH is not able to increase estrone 3-sulfate binding to the mutated MRP1. Collecting results indicate that TM6-Lys332 mutant

impairs GSH-stimulated estrone 3-sulfate transport via losing GSH binding, while TM17-Trp1246 mutant impairs transport due to a loss of binding of sulfated estrogen. All these results gave the conclusion that TM17-Trp1246 is critical for recognition of methotrexate, $E_2$17βG, and estrone 3-sulfate by MRP1, whereas TM6-Lys332 is critical for recognition of GSH and compounds such as LTC4 that contain a GSH moiety. However, inhibitory experiment demonstrates that neither TM17-Trp1246 nor TM6-Lys332 is critical for recognition of the non-GSH containing modulators MK571, BAY u9773, and LY171883. TM17-Trp1246 seems to be important for recognition of the tricyclic isoxazole derivative LY465803.

Analysis of differences in the substrate and inhibitor selectivity of MRP1 mutants has led to the conclusion that MRP1 contains at least three classes of substrate/modulator-binding sites: one that requires TM17-Trp1246, one that requires TM6-Lys332, and a third that requires neither of these residues. TM17-Trp1246 is important for conferring drug resistance and for transport of methotrexate, $E_2$17βG, and estrone 3-sulfate, as well as for binding of tricyclic isoxazole inhibitors such as LY465803. TM6-Lys332 is crucial for enabling GSH and GSH-containing compounds to serve as substrates or modulators of MRP1 and, further, for enabling GSH to enhance the transport of estrone 3-sulfate and increase the inhibitory potency of LY465803. Nevertheless, neither Trp1246 nor Lys332 is important with respect to the activity of the non-GSH-containing small molecule inhibitors MK571 and LY171833 or BAY u9773 (Maeno et al. 2009).

So far, no good inhibitors of MRP1 are developed, although a variety of inhibitors of MRP1 have been described. Some examples are MK571, S-decylglutathion, sulfinpyrazone, benzbromarone, and probenecid. However, most of them poorly enter cells; it is difficult to obtain sufficient intracellular concentration for efficacious inhibition. Some P-GP inhibitors such as cyclosporin A, PSC 833, with reasonable cellular penetration, do inhibit MRP1 but only with low affinity and poor specificity. These inhibitors are not suitable for in vivo study; they extensively affect functions of other transporters and need to be used at relatively high concentration.

2.3.1.3 Pharmacological Functions of MRP1

Roles of MRP1 in human health and disease have been confirmed. MRP1 mediates efflux of many antineoplastic agents such as vincristine, doxorubicin, and methotrexate, inferring contribution of MRP1 to tumor multidrug resistance in clinical oncology. Overexpression of MRP1 has been associated with poor patient outcome in breast cancer (Filipits et al. 2005), NSCLC (Li et al. 2010), acute myeloid leukemia (AML) (Schaich et al. 2005), and ALL (Plasschaert et al. 2005). Overexpression of MRP1 expression is strongly predictive of poor outcome in neuroblastoma patient cohorts (Haber et al. 2006; Henderson et al. 2011) and is served as the major prognostic indicators in this disease. Furthermore, MRP1 is transcriptionally regulated by the MYCN oncogene (Porro et al. 2010) whose amplification occurs in approximately 20–30% of primary neuroblastomas and is

consistently associated with poor clinical outcome (Brodeur et al. 1984; Seeger et al. 1985). Henderson et al. (2011) also reported that disruption of MRP1 decreases tumor incidence and increased tumor latency in the MYCN transgenic mouse model of neuroblastoma. Now, considerable efforts have been made to develop ways to improve chemotherapy effectiveness in cancer patients via overcoming drug resistance.

MRP1 also plays a vital role in the efficacy (and toxicity) of drugs used to treat nonmalignant diseases. A report demonstrated that *Abcc1*$^{-/-}$ mice are more sensitive to the toxicity of intravenously administered etoposide in the oropharyngeal mucosal layer and testicular tubules (Wijnholds et al. 1998) than wild-type mice. Generally MRP1 and other ABC transporters (including P-GP and MRP2) commonly protect tissue from drug-induced toxicity. In mice, combination of deficiency of *Mdr1a/1b*$^{-/-}$:*Abcc1*$^{-/-}$ resulted in a dramatically increased sensitivity to intraperitoneally administered vincristine (up to 128-folds), also to etoposide (3–5 folds), whereas deficiencies of *mdr1a/1b*$^{-/-}$ and *Abcc1*$^{-/-}$ alone only exhibited 16- and 4-fold increase in toxicity to vincristine, respectively (Johnson et al. 2001). An in vitro assay showed that bone marrow of *Mdr1a/1b*$^{-/-}$, *Abcc1*$^{-/-}$, and *Mdr1a/1b*$^{-/-}$:*Abcc1*$^{-/-}$ mice sensitivity to vincristine was 2-, 5- to 10-, and 25-fold of wild-type mice, respectively (van Tellingen et al. 2003). Although MRP1 and P-GP are highly expressed at BBB, Wang et al. (2010a) reported that ratio of brain-to-plasma vincristine concentrations in *Mdr1a/1b*$^{-/-}$:*Abcc1*$^{-/-}$ mice were greater than those in wild-type mice. *Abcc1*$^{-/-}$ mice and *Mdr1a/1b*$^{-/-}$: *Abcc1*$^{-/-}$ mice also showed greater ratio of brain tumor-to-plasma vincristine concentrations than wild-type mice. Comparisons of vincristine concentrations within each strain indicated that vincristine brain tumor concentrations were statistically greater than either brain, ranging from 9- to 40-fold. These results infer that contribution of MRP1 to efflux of vincristine seems to be larger than that of P-GP, which is consistent with the findings that bone marrow of *Mdr1a/1b*$^{-/-}$ mice was less sensitive to vincristine than that of *Abcc1*$^{-/-}$ mice (van Tellingen et al. 2003). Similarly, Wijnholds et al. (2000) reported that etoposide concentration in the cerebrospinal fluid of *Mdr1a/1b*$^{-/-}$: *Abcc1*$^{-/-}$ mice was about tenfold of *Mdr1a/1b*$^{-/-}$ mice after intravenous administration.

MRP1 is well known to play an important role in cellular protection against inorganic arsenicals. Exposure of inorganic arsenic (arsenate [As^{V}] and arsenite [As^{III}]) may induce many diseases such as tumor, peripheral vascular disease, neurological disorders, and diabetes mellitus. People are often exposed to As^{V} and As^{III} via drinking water or food. Arsenic trioxide (As_2O_3) has been approved for treating acute promyelocytic leukemia (Emadi and Gore 2010). Inorganic arsenic is biomethylated in a sequence of enzymatically catalyzed reactions that produce methylarsenic (MMAs), dimethylarsenic (DMAs), and trimethylarsenic (TMAs) species, which contain either trivalent arsenic (As^{III}) or pentavalent arsenic (As^{V}). The trivalent forms MMA^{III} and DMA^{III} are more reactive and toxic than arsenite (As^{III}) and arsenate (As^{V}). In presence of glutathione transferase P1, As^{III}, and MMA^{III} are conjugated with GSH to form triglutathione conjugate of arsenite [$As^{III}(GS)_3$] and diglutathione conjugate of MMA^{III}[$MMA^{III}(GS)_2$]. $MMA^{III}(GS)_2$ and $As^{III}(GS)_3$ are substrates of MRP1, inferring that MRP1 is an important cellular

protective pathway for the highly toxic MMA^{III} and has implications for environmental and clinical exposure to arsenic (Leslie 2012; Carew et al. 2011).

In addition to protecting the body against drugs, environmental toxins, and heavy metals, MRP1 has been implicated in the etiology of a wide array of human pathologies. 4-hydroxy-nonenal is a relatively stable α,β-unsaturated electrophile product by the peroxidation of polyunsaturated fatty acids in tissues under oxidative stress. Transport of its GSH conjugate is also mediated by MRP1. MRP1 transport has a chemoprotective role in multiple tissues, which is verified by several reports that the occurrence of adverse drug reactions is associated with certain ABCC1 SNPs (Vulsteke et al. 2015; Semsei et al. 2012). An important example is doxorubicin cardiotoxicity in adult and pediatric cancer patients, which results from overproduction of reactive oxygen species and oxidative stress. Mouse study also confirmed that loss of Mrp1 potentiated doxorubicin-induced cytotoxicity (Zhang et al. 2015).

MRP1 is highly expressed in endothelial cells and hematopoietic stem cells. Several studies have demonstrated that MRP1 blockade prevents endothelial cell apoptosis and improves endothelial function. It was reported that MRP1 inhibitor MK571 increased intracellular GSH levels, reduced intracellular reactive oxygen species levels in endothelial progenitor cells, prevented angiotensin II-induced apoptosis, and increased the number of early outgrowth endothelial progenitor cells and colony-forming units. These results were further confirmed using *Abcc1*$^{-/-}$ mice (Mueller et al. 2010). In consistence, effects of angiotensin II on vessels, such as hypertension, increase in superoxide production, and decrease in aortic tetrahydrobiopterin, were markedly blunted in *Abcc1*$^{-/-}$(Widder et al. 2007), inferring that MRP1 is involved in the genesis of multiple vascular abnormalities that accompany hypertension and that MRP1 presence is essential for the hypertensive response to angiotensin II.

Sphingosine-1-phosphate and lysophosphatidylinositol are substrates of MRP1. The two molecules participate in a myriad of cellular processes mostly through their actions on G protein-coupled receptors (GPCRs), and disruptions in their signaling pathways have been implicated in an array of inflammatory, cardiovascular, neurological, and malignant diseases. The mechanism that they can act on their target receptors is poorly understood, although it seems likely that multiple cell type-specific mechanisms are involved. Mitra et al. (2006) reported that knocking down MRP1 with *ABCC1* siRNA and MK571 both decreased export of sphingosine-1-phosphate from mast cells. Several studies have indicated that Mrp1 mediates sphingosine-1-phosphate efflux from rat uterine leiomyoma cells and mouse adipocytes (Tanfin et al. 2011; Ito et al. 2013). A role for Mrp1 in sphingosine-1-phosphate-mediated signaling was also demonstrated using the brain and spinal cord capillaries isolated from *Abcc1*$^{-/-}$ mice (Cartwright et al. 2013). Another signaling molecule lysophosphatidylinositol, associated with decreased proliferation and release from PC-3 human prostate cancer cells, was reported to reduce by 50% following downregulation of MRP1 using siRNA (Piñeiro et al. 2011).

Cysteinyl leukotrienes (CysLTs) such as LTC_4, LTD_4, and LTE_4, potent pro-inflammatory molecules, are also substrates of MRP1. LTC_4 is derived from

arachidonic acid via 5-lipoxygenase/leukotriene C4 synthase pathway and excreted from mast cells and is rapidly converted to LTD_4 and then to LTE_4. CysLTs have roles in both innate and adaptive immune processes. In the airways, they induce smooth muscle contraction and increase vascular permeability and mucus secretion, inferring that CysLTs are crucial mediators in the pathogenesis of allergic asthma and that MRP1 implicates the development of allergic airway disease via regulating CysLT transport. Mouse studies confirmed that the *Abcc1*$^{-/-}$ mice exhibited lower airway inflammation and goblet cell hyperplasia following exposure of ovalbumin than wild-type mice, which was consistent with significant decreases in levels of CysLTs, IgE, IL-4, and IL-13 in bronchoalveolar lavage fluid. IgE-mediated CysLT release from bone marrow-derived mast cells of *Abcc1*$^{-/-}$ mice was also significantly decreased (Yoshioka et al. 2009), indicating that MRP1 inhibitors may be useful in the treatment of inflammatory disorders, including those involving the airways such as asthma and chronic obstructive pulmonary disease (COPD) (Yoshioka et al. 2009). Unfortunately, clinical reports are often conflicting. A report showed that MRP1 expression was lower in bronchial biopsies of chronic COPD patients than that of healthy controls and was also lower in patients with severe COPD than with mild/moderate COPD (van der Deen et al. 2006). In COPD patients using placebo, or inhaled corticosteroids with or without long-acting β2-agonists, Budulac et al. (2012) reported that subjects with a moderate staining for MRP1 had less forced expiratory volume decline than those with a weak staining during 30-month inhaled corticosteroid treatment. On the contrast, subjects stopping inhaled corticosteroids after 6 months followed by 24-month placebo and moderate staining for MRP1 were associated with faster forced expiratory volume decline than in those with a weak staining. Several genomic studies have indicated that certain ABCC1 polymorphisms may be associated with greater or lesser severity of chronic obstructive pulmonary disease, as well as possibly response of asthma (Siedlinski et al. 2009; Budulac et al. 2010). No association between MRP1 variants and forced expiratory volume decline was also reported (Budulac et al. 2012).

*2.3.2 Multidrug Resistance Protein 2 (MRP2/*ABCC2*)*

2.3.2.1 General Properties and Distribution of MRP2

The multidrug resistance protein 2 (MRP2/*ABCC2* for human and Mrp2/*Abcc2* for animals), 190 kDa protein, is the second member of the subfamily of MRP efflux pumps encoded by *ABCC2* gene, which was once termed as cMOAT or cMRP (canalicular multispecific organic anion transporter). The Mrp2 was first cloned from rat liver in 1996 using a strategy that took advantage of its sequence similarity to human MRP1. Soon thereafter, the human, rabbit, mouse, and canine orthologues were cloned and, as for all of the MRP2/Mrp2, show a high degree of amino acid identity with one another (77–83%). MRP1 and MRP2 belong to the same ABC subfamily, sharing 50% amino acid identity (Nies and Keppler 2007). The size and

topology of MRP2 are similar to MRP1, but MRP2 contains additional sequence motifs in the cytoplasmic region linking TMD to TMD1, a lysine-rich element, that is essential for apical targeting (Bandler et al. 2008). MRP2 is strategically located at the apical membrane of polarized cells anchored to the actin cytoskeleton via ezrin/radixin/moesin (ERM) family proteins. MRP2 and ERM can interact to each other directly or via postsynaptic density 95/disc-large/zona occludens (PDZ) proteins, which is crucial for location, stability, and activity of MRP2 (Arana et al. 2016). Site mutagenesis indicates that MRP2 contains two similar but nonidentical ligand-binding sites: one site from which substrate is transported and a second site that regulates the affinity of the transport site for the substrate (Zelcer et al. 2003).

It is different from MRP1, and most of other MRPs, that MRP2 is located at the apical membrane of polarized epithelial and endothelial cells, predominantly those in the liver, kidney, and intestine (Slot et al. 2011; Fardel et al. 2005). In the liver, MRP2 is primarily expressed at canalicular membrane of hepatocytes. In the kidney, MRP2 is expressed at the apical membranes of the proximal tubule of the kidney. MRP2/Mrp2 is expressed in the brush border membrane domain of segments S_1, S_2, and S_3 of proximal tubule epithelia in rat kidney and in proximal tubules of human kidney. In the small intestine of rats, Mrp2 expression is concentrated at the tip of the villus, and obviously regional-dependent Mrp2 expression is found throughout the small intestine. Expression of Mrp2 decreases from the proximal to distal regions of the intestine, which is opposite to that of P-GP (Dahan et al. 2009). The distribution of MRP2 along the small intestine, the primary site of absorption of orally administrated xenobiotics, agrees well with phase II conjugating enzymes (UDP-glucuronosyltransferase and glutathione *S*-transferase), suggesting that metabolism and subsequent efflux of the organic anion conjugates act coordinately to decrease the intestinal absorption of drugs and harmful compounds.

MRP2 is also localized to the luminal membrane of brain microvascular endothelial cells and apical syncytiotrophoblast membrane of the term placenta. In analogy with P-GP present at these locations, MRP2 may limit the brain and fetal penetration of a range of substrate compounds. Overall, there is fairly extensive overlap between MRP2 and P-GP tissue distribution, inferring it is likely that the two proteins have considerable overlap in pharmacological and toxicological protective functions, albeit with different sets of substrates (Schinkel and Jonker 2012). In addition to normal tissues, the MRP2 is also present in a number of human malignant tumors such as renal clear-cell, hepatocellular, ovarian, colorectal, lung, breast, and gastric carcinomas (Nies and Keppler 2007). Some anticancer drugs are also MRP2 substrates, inferring that MRP2 may clinically contribute to the multidrug resistance phenotype of several solid malignant human tumors.

It is noted that despite the high level of identity between human MRP2 and other species Mrp2, the marked species differences in MRP2/Mrp2 transporter activity have been reported in both in vitro and in vivo models (Ishizuka et al. 1999; Zimmermann et al. 2008). For example, Ishizuka et al. (1999) found that the in vitro transport of 2,4-dinitrophenyl-*S*-glutathione into canalicular membrane vesicles was eightfold higher (V_{max}/K_m: 64.2 versus 7.7 μl/min/mg protein) in rat than in dog, whereas the in vivo biliary excretion of temocaprilat was 40-fold higher

in rat than that in dog. In consistence, expression of hepatic Mrp2 in rat liver was approximately tenfold higher than that in other species (Li et al. 2009). Data from MDCK cells stably expressing human MRP2 or mouse Mrp2 demonstrated that saquinavir and docetaxel were more efficiently transported by mouse Mrp2, whereas vinblastine was transported better by human MRP2. MRP2 modulator sulfinpyrazone notably stimulated $E_2$17β transport by human MRP2 but profoundly inhibited mouse Mrp2 activity (Zimmermann et al. 2008). These differences should be taken into account when results obtained in rats are extrapolated to humans.

2.3.2.2 Substrates and Inhibitors of MRP2

The nature of MRP2 substrates is very close to MRP1. MRP2 mediates the ATP-dependent transport of various organic anions, most of which are their glucuronate, sulfate, or GSH conjugates (Table 2.5). Some anionic substances without anionic conjugate residues are also MRP2 substrates, such as methotrexate, pravastatin, and bromosulfophthalein. Like MRP1, MRP2 effluxes some substrates such as vincristine and daunorubicin via a cotransport mechanism with GSH, which is evidenced by the fact that vinblastine was transported with GSH transport and depletion of cellular GSH by treatment with L-buthionine sulfoximine resulted in decreased substrate transport and drug resistance in MRP2-overexpressing cells. The spectrum of MRP2 substrates is often similar to MRP1, but there is not a complete overlap. One important difference between MRP1 and MRP2 is that overexpression of MRP2, but not MRP1, is associated with cisplatin resistance (Leslie et al. 2005). In addition, some MRP2 substrates may be substrates of other transporters such as P-GP and other MRPs.

Many of MRP2 substrates are also identified to be competitive inhibitors of MRP2 using inside-out membrane vesicles. Some examples are LTC_4, MK571, phenolphthalein glucuronide, and fluorescein methotrexate. However, these compounds frequently do not penetrate most normal cells to a sufficient extent to obtain useful levels of inhibition. They are not suitable for in vivo study. Other

Table 2.5 Some clinically relevant transported substrates of MRP2

Endogenous compounds	LTC4, LTD4, LTE4, ($E_2$17βG), bilirubin and its glucuronidated conjugates, bile acids, GSSG
Anticancer drug	Methotrexate, doxorubicin, epirubicin, mitoxantrone, vincristine, vinblastine, irinotecan, SN-38, etoposide, chlorambucil, cyclophosphamide, cisplatin, oxaliplatin, As_2O_3.
HIV protease inhibitor	Adefovir, cidofovir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir
Others	p-aminohippurate, S-glutathionyl 2,4-dinitrobenzene, pravastatin, (S-glutathionyl-)sulfobromophthalein, sulfinpyrazone, catechol conjugates with GSH, azithromycin, Fluo-3, acetaminophen conjugates with glucuronide, sulfate, GSH, olmesartan, eprosartan, temocaprilat
Toxins	Bisphenol A, ochratoxin A, food-derived (pre-)carcinogens, Hg^{2+}

compounds such as cyclosporin A, sulfinpyrazone, benzbromarone, probenecid, glibenclamide, rifampicin, montelukast, and indomethacin, to a greater or lesser extent, inhibit MRP2 activity, although they may also interact with other transporters including other MRPs, P-GP, organic anion-transporting polypeptides (OATPs), or BSEP.

2.3.2.3 Pharmacological and Toxicological Functions of MRP2

The in vivo function of MRP2/Mrp2 is well studied in patients suffered from the Dubin-Johnson syndrome and in two mutant rat strains: the *GY/TR*$^{-}$ rats and the Eisai hyperbilirubinemic rats (EHBR). These patients are unable to excrete glucuronidated bilirubin into the bile and consequently develop a permanent conjugated hyperbilirubinemia due to mutations of *ABCC2* gene such as splice-site mutations, missense mutations, and nonsense mutations. The two rat strains, sequence variants of *Abcc2* gene occurring either at codon 401 for *GY/TR*− rats or at codon 855 for EHBR, leading to premature stop codons and to a lack of the Mrp2 protein at the hepatocyte canalicular membrane, cannot mediate the hepatobiliary excretion of glucuronidated bilirubin into bile. The two mutant rat strains can be considered as animal models of human Dubin-Johnson syndrome (Schinkel and Jonker 2012). Several studies have also supported important roles of hepatic MRP2/Mrp2 in the development of acquired and hereditary jaundice. Sepsis, inflammatory cholestatic disease (such as alcoholic hepatitis and chronic hepatitis C), and obstructive cholestasis are all associated with a decrease in canalicular MRP2/Mrp2 expression in rodents and conjugated hyperbilirubinemia (Cuperus et al. 2014). Hepatic MRP2/Mrp2 also transports GSH and bile acids. MRP2-mediated GSH transport helps to create an osmotic gradient in the bile canalicular lumen and is mainly responsible for the instigation of the bile acid-independent bile flow. The facts that the bile flow in the *Abcc2*$^{-/-}$ mice was reduced to approximately 37% of wild-type mice and that plasma level of total bilirubin was increased to b 2–3-fold of wild-type mice further support above deduction (Vlaming et al. 2006). MRP2 and other transporters overlap substrate specificity, inferring that *ABCC2* deficiency is partly compensated by the activity of alternative transporters, which may be responsible for the absence of a severe hepatic phenotype in Dubin-Johnson patients. Data from mouse study demonstrated that effect of common knockout *Mdr1a/b* and *Abcc2* on biliary excretion of doxorubicin is stronger than single knockout *Mdr1a/1b* or *Abcc2*. It was reported that the excretion of doxorubicin in the *Mdr1a/1b*$^{-/-}$:*Abcc2*$^{-/-}$ mice was 5-fold lower and 26-fold lower than that in the *Mdr1a/1b*$^{-/-}$ mice and in the *Abcc2*$^{-/-}$ mice, respectively, indicating that *Abcc2* and *Mdr1a/1b* can partly compensate for the absence of the other ABC transporter (Vlaming et al. 2006). Similarly, effects of single knockout *Abcc2* or *Abcg2* on plasma exposure of methotrexate following oral and intravenous administration of methotrexate are less than double knockout *Abcc2*$^{-/-}$:*Abcg2*$^{-/-}$. *Abcc2*$^{-/-}$:*Abcg2*$^{-/-}$ mice showed higher plasma exposure of methotrexate (Vlaming et al. 2011).

MRP2 also transports a wide range of xenobiotic substrates and their conjugates with glucuronic acid, glutathione, and sulfate, most of which are therapeutic drugs, inferring that MRP2 activity affects the pharmacokinetics of many therapeutic agents via intestinal absorption and biliary excretion or urinary excretion. One of the main functions for intestinal MRP2 is to limit oral bioavailability, leading to decreased therapeutic efficacy of MRP2 substrates of clinical use. It is noteworthy that food contaminants of toxicological relevance like ochratoxin A and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) are also transported by MRP2. A report demonstrated that plasma concentrations of two food-derived carcinogen, PhIP and 2-amino-3-methylimidazo [4,5-f]quinolone, were 1.9- and 1.7-fold higher in *Abcc2*$^{-/-}$ mice than those in wild-type mice, respectively, demonstrating the role of Mrp2 in restricting exposure to these compounds (Vlaming et al. 2006).

Mrp2 also exists in the luminal membrane of rat brain microvessel endothelial cells. Human MRP2 is expressed in apical syncytiotrophoblast membrane of the term placenta. In analogy with P-GP present at these locations, MRP2 may limit the brain and fetal penetration of a range of substrate compounds present in (maternal) plasma. Overall, there is fairly extensive overlap between MRP2 and P-GP tissue distribution, so it is likely that these two proteins have considerable overlap in pharmacological and toxicological protective functions, albeit with different sets of substrates.

Like MRP1, MRP2 also acts synergistically with several phase II conjugating enzymes including the GSH S-transferases and UDP-glucuronosyltransferases to confer resistance to the toxicities of several electrophilic drugs and carcinogens. MRP2 polymorphisms occur in a higher frequency in patients with nonfatty alcoholic liver disease, intrahepatic cholestasis of pregnancy, bile duct cancer, and diclofenac- induced hepatotoxicity. MRP2 polymorphisms also lead to a decreased biliary excretion of toxic metabolites during irinotecan treatment, which protects patients from irinotecan-induced diarrhea (de Jong et al. 2007). Methotrexate is a key agent for the treatment of childhood ALL. Increased methotrexate plasma concentrations are associated with a higher risk of adverse drug effects. Patients with the 224 T allele in MRP2 gene was reported to be significantly associated with higher risks of high-grade hematologic (leucopenia, anemia, and thrombocytopenia) and non-hematologic (gastrointestinal and mucosal damage/oral mucositis) methotrexate toxicities, accompanied by significantly higher methotrexate plasma concentrations at 48 h after the start of infusion (Liu et al. 2014).

In vitro study well demonstrates involvement of MRP2 in several cancer cell cross-resistance to various anticancer drugs such as methotrexate, epipodophyllotoxins, vincristine, doxorubicin, and cisplatin. Oxaliplatin and its anionic monochloro oxalate ring-opened intermediate are also MRP2 substrates (Myint et al. 2015). MRP2 is highly expressed in some human cancerous tissues. All these infer MRP2 contribution to clinical anticancer drug resistance. It was reported that that MRP2 expression determined the efficacy of cisplatin-based chemotherapy in patients with hepatocellular carcinoma (Korita et al. 2010). Similarly, MRP2-positive immunostaining was reported to be more frequently observed in esophageal squamous cell carcinoma (ESCC) with neoadjuvant chemotherapy

(NACT) than in those without NACT (27.3% versus 5.4%). The MRP2-positive patients showed poorer prognosis than MRP2-negative patients (5-year survival rate, 25.6% versus 55.7%). ESCC with NACT showed 2.1-fold higher mRNA expression of MRP2 than those without NACT. In pre-therapeutic biopsy samples of patients with NACT, non-responders showed 2.9-fold higher mRNA expression of MRP2 than responders. ESCC cell line data further demonstrated strong association of MRP2 mRNA expression with the resistance to cisplatin (Yamasaki et al. 2011). In patients with small-cell lung cancer, the response rate in the MRP2-negative group was significantly higher than that in the MRP2-positive group (88% versus 50%). Results from 37 patients treated with platinum-based chemotherapy showed that the response rate of patients in the MRP2-negative group was significantly higher than that in the positive group (92% versus 50%). These results suggest that immunostaining of MRP2 for transbronchial biopsy specimens may help to predict clinical resistance to platinum agents (Ushijima et al. 2007).

Like MRP1, MRP2 plays an important role in cellular protection against inorganic arsenicals (Leslie 2012). In addition, MRP2 also transports other toxic metals such as platinum (Shord et al. 2006; Wen et al. 2014), mercury (Engström et al. 2013), and cadmium (Carrière et al. 2011). In human, platinum mainly results from anticancer platinum drugs such as oxaliplatin and cisplatin. Their GSH conjugates are MRP2 substrate. An estimated 20% of patients receiving high-dose cisplatin have severe renal dysfunction, and approximately one-third of patients experience kidney injury just days following initial treatment (Shord et al. 2006), due to local accumulation of cisplatin inside the proximal tubule. Two different membrane transporters, copper transporter 1 and organic cation transporter 2, are considered to be able to facilitate the transport of cisplatin into cells. In the kidney, cisplatin undergoes metabolic activation in the kidney to a more potent nephrotoxin cisplatin-GHS conjugates via glutathione-S-transferase or γ-glutamyltranspeptidase. The $Pt(GS)_2$ complex was effluxed out of via GX pumps (including MRP2) (Fig. 2.4), inferring roles of MRP2 in detoxifying cisplatin toxicity (Oh et al. 2016; Chen and Kuo 2010). It was reported that the kidneys and livers of *Abcc2*$^{-/-}$ mice showed twofold higher concentrations of platinum than wild-type mice, accompanied by significant increase in cisplatin nephrotoxicity. These alterations in *Abcc2*$^{-/-}$ mice were almost reversed by transgenic expression of the human *ABCC2* gene (Wen et al. 2014).

MRP2 plays roles in disposition and elimination of mercury. Animal experiments demonstrated that compared with wild-type animals, both *TR*$^{-}$ rats and *Abcc2*$^{-/-}$ mice (Bridges et al. 2013) exhibited the higher renal accumulation of Hg^{2+}, especially in the renal cortex. Moreover, *TR*$^{-}$ rats showed significantly lower urinary excretion of Hg^{2+}. These results confirmed that the lack of Mrp2 contributes to the enhanced accumulation of Hg^{2+} in the epithelial cells of the proximal tubule (Zalups et al. 2014). MRP2 also affect placental and fetal disposition of mercuric ions. The amount of mercury in the renal tissues, liver, blood, amniotic fluid, uterus, placentas, and fetuses of *TR*$^{-}$ rats following exposure of a single dose of methylmercury was reported to be significantly greater than that in Wistar rats. Urinary and fecal elimination of mercury in *TR*$^{-}$ rats was less than that in Wistar rats (Bridges et al.

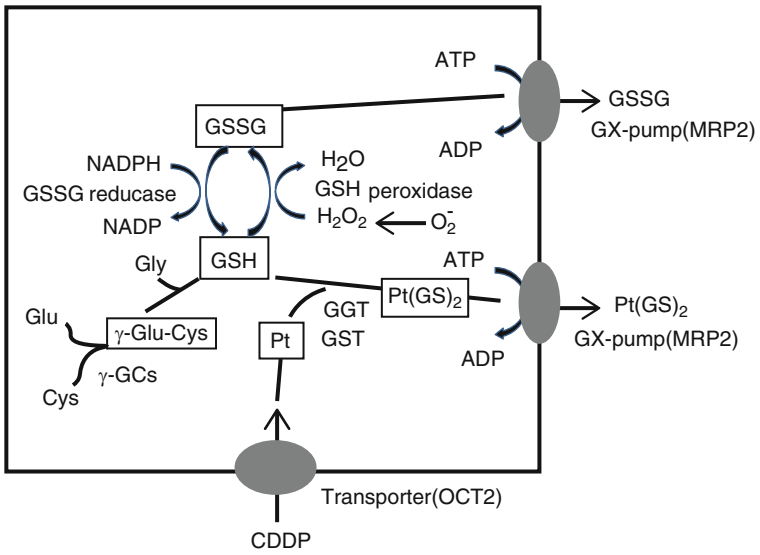

Fig. 2.4 The role of GSH and MRP2 in MRP-mediated CDDP (cisplatin) transport. *GST* glutathione-S-transferase, *GGT* γ-glutamyltranspeptidase, *Glu* glutamine, *Cys* cysteine, *Gly* glycine, *Pt (GS)2* platinum conjugates with GSH, *OCT2* organic cation transporter 2, *γ-GCs* γ-glutamylcysteine synthetase

2012). A clinical investigation (Engström et al. 2013) on populations exposed to mercury vapor from gold mining showed that *ABCC2* SNPs was associated with high urinary Hg^{2+} concentrations. Urinary Hg concentrations in *ABCC2 rs1885301* A allele carriers were higher than that in GG homozygotes, though differences were not statistically significant. Three *ABCC2* SNPs showed particularly strong associations with urinary Hg. Subjects carrying A allele of *rs1885301* had higher urinary Hg than GG homozygotes, carrier of *rs2273697 GG* homozygotes had higher urinary Hg than A allele carriers, and carriers of *rs717620* A allele had higher urinary Hg than GG homozygotes.

Several studies have demonstrated that MRP2 can also be located in the nuclear membrane of cancer cell, specifically for lower differentiated cells and stem cells. The alterations in MRP2 location may affect cell sensitivity to chemotherapeutical drugs and their clinical outcome. Halon et al. (2013) reported that nuclear membrane location of MRP2 can be associated with lower differentiation of cancer cells and their resistance to the cisplatin. Similar report also demonstrated association of nuclear MRP2 expression with response to first-line chemotherapy for ovarian carcinomas. Cases with relapse showed higher nuclear membranous localization of MRP2 expression. Weak nuclear membranous localization of MRP2 was associated with significantly longer overall and progression-free survival. In ovarian carcinoma cells, nuclear membranous localization of MRP2 correlated with resistance against cisplatin, whereas localization in the cytoplasmic membrane did not (Surowiak et al.

2006). In addition, higher nuclear MRP2 expression was identified in breast cancer from patients, which was associated with a shorter disease-free survival and a shorter progression-free time. Cases with nuclear expression of MRP2 manifested a more aggressive clinical course, which might reflect a less advanced differentiation of neoplastic cells, resistance to the applied cytostatic drugs, and tamoxifen (Maciejczyk et al. 2012).

2.3.3 Multidrug Resistance Protein 3 (MRP3/ABCC3)

2.3.3.1 General Properties of MRP3

MRP3/Mrp3 (MRP3/*ABCC3* for human and Mrp3/*Abcc3* for animals) consists of 1, 527 amino acids (weight of 190–200 kDa) and is the closest in structure to MRP1, with 56% amino acid identity (Slot et al. 2011). Like MRP1, MRP3 is expressed on the basolateral membranes of polarized cells. In human, MRP3 is expressed mainly in the liver, adrenal gland, placenta, testis, intestine, colon, and gallbladder and at a relatively lower level in the pancreas, kidney, lung, and tonsils (Wang et al. 2010b; Slot et al. 2011). In the intestine of rats, the expression of intestinal Mrp3 is the highest in the colon, whose expression is almost equal in other segments (Kitamura et al. 2008). Hepatic expression of MRP3 has been linked to the concentration of serum bilirubin or its glucuronides, suggesting that these endogenous compounds may induce expression of MRP3 (Ogawa et al. 2000). Although normal brain tissue lacks MRP3 expression, overexpression of MRP3 (Kuan et al. 2010) occurs in glioblastoma multiforme. The expression of MRP3 has been identified to be glioblastoma multiforme-associated molecule and implicated as one of the cell surface targets for glioblastoma multiforme (Loging et al. 2000). The elevated MRP3 mRNA levels in glioblastoma multiforme biopsy samples were reported to be correlated with a higher risk of death (Kuan et al. 2010). MRP3 is also expressed in cell lines such as human glioblastoma multiforme-derived cells (Kuan et al. 2010), primary NSCLC cells, and metastasis-derived primary NSCLC cells (Melguizo et al. 2012) as well as hepatocellular carcinoma cells (Tomonari et al. 2016). In addition, overexpression of MRP3 was reported in astrocytomas as the primary resistance to chemotherapy with drugs like cis-platinum and carmustine (Calatozzolo et al. 2005). Although MRP3 is much less expressed in cancer stem cells, differentiation notably raised MRP3 expression, suggesting that the differentiated cells also acquire chemotherapeutic resistance via MRP3 (Jin et al. 2008).

MRP3 is a quite broad specificity organic anion transporter, with considerable overlap in drug substrates with other transporters including MRP1 and MRP2. It is generally accepted that MRP3 is involved in the cellular extrusion of organic anions including monovalent bile acids (taurocholate and glycocholate). Glucuronate conjugates are preferred substrates for MRP3, whereas nonconjugated organic anions (such as methotrexate), bile acid sulfates, and glutathione conjugates are poor substrates of MRP3. Despite its sequence similarity to MRP1 and MRP2, MRP3 has a

very different substrate profiles. The most striking difference is the very low affinity and capacity of MRP3 to transport GSH (Borst et al. 2007). Unlike MRP1 and MRP2, MRP3 also does not require GSH for efflux of etoposide (Zelcer et al. 2001). Cells expressing MRP3 are significantly resistant to etoposide and teniposide, but not to vincristine, doxorubicin, and cis-platinum (Zelcer et al. 2001; Grant et al. 2008). MRP3 also displays complex transport kinetics with substrates. Seelheim et al. (2013) reported cooperative interaction of two subunits for MRP3 during substrate translocation and stimulating ATP hydrolysis. The two subunits showed identical apparent K_m values for the tested substrates $E_2$17βG, LTC4, and methotrexate. $E_2$17βG, LTC4, and methotrexate stimulated ATP hydrolysis in a positive allosteric cooperative manner, whose Hill coefficients were near to 2. Similarly, transport of the above three substrates by MRP3 also was also positive allosteric cooperative. However, 5(6)-carboxy-2′-7′-dichlorofluorescein, another MRP3 substrate, did not stimulate ATPase activity, although its transport by MRP3 was also positive allosteric cooperative. All these suggest that transport and ATPase activity of MRP3 are half-coupled (Seelheim et al. 2013).

A number of classical organic anion transport inhibitors, such as benzbromarone, indomethacin, probenecid, and sulfinpyrazone, may reverse the MRP3-mediated decrease in etoposide accumulation. Unlike MRP2, the major physiological role of MRP3 is to transport conjugated metabolites back to the bloodstream. In addition, effects of bile acids and organic anions on the transport $E_2$17βG by MRP3 are different from that of MRP2. Organic anions indomethacin, furosemide, and probenecid as well as conjugated bile acids significantly stimulate $E_2$17βG transport by MRP2. On the contrast, all these agents inhibit transport of $E_2$17βG by MRP3 (Bodo et al. 2003).

2.3.3.2 Pharmacological and Physiological Functions of MRP3

MRP3 is primarily located on the basolateral membrane of hepatocytes and enterocyte. An attractive possibility is that MRP3 may play a role in the cholehepatic and enterohepatic circulation of bile salts. A study using membrane vesicles from LLC-PK1 cells expressing rat Mrp3 confirmed that endogenous bile salts including taurocholate, glycocholate, taurochenodeoxycholate-3-sulfate and taurolithocholate-3-sulfate are substrates of rat Mrp3 (Hirohashi et al. 2000). Rat liver perfusion experiments also demonstrated both the contribution of Mrp3 to the basolateral excretion of hyodeoxycholate-glucuronide (Zelcer et al. 2006) and the positive correlation of sinusoidal efflux clearance of taurocholate with the hepatic expression of Mrp3 (Akita et al. 2001). Interestingly, in human, the expression of hepatic MRP3 is very low, but it was induced in patients with Dubin-Johnson syndrome and patients with primary biliary cirrhosis (Konig et al. 1999). Similarly, hyperbilirubinemia patients in intensive care unit possessed the elevated conjugated cholic acid and chenodeoxycholic acid compared with control patients. The levels of hepatic MRP3 mRNA and protein were strongly upregulated, which positively correlated to both degree of bilirubinostasis and biochemical biomarkers of

cholestasis liver dysfunction (Vanwijngaerden et al. 2011). In rats, *Abcc2* deficiency (Akita et al. 2001, 2002) or cholestasis (Kamisako and Ogawa 2005; Hasegawa et al. 2009) also upregulated of hepatic Mrp3 expression. These results infer the roles of MRP3 in bile acid homeostasis via affecting enterohepatic circulation of bile salts. However, several reports showed that bile acid homeostasis was unaltered in *Abcc3*$^{-/-}$ mice. No differences were observed in histological liver damage and serum bile salt levels between *Abcc3*$^{-/-}$ mice and wild-type mice (Belinsky et al. 2005; Zelcer et al. 2006) following bile duct ligation. Moreover, *Abcc3*$^{-/-}$ mice had no abnormalities in taurocholate uptake in the ileum, whose the total fecal bile salt excretion was similar to that of wild-type mice (Zelcer et al. 2006; Belinsky et al. 2005), inferring that roles of Mrp3 in the enterohepatic circulation of bile salts were minor, at least in mice. In fact, many transporters including apical sodium-dependent bile acid transporter (ASBT), organic anion-transporting peptides (OATPs), and organic solute transporters (OST) involve intestinal reabsorption of the secreted bile acids (Alrefai and Gill 2007). Different from murine Mrp3, human MRP3 only mediates transport of the monovalent glycocholic bile acid, but taurocholate is poor substrate of human MRP3 (Zeng et al. 2000)**.** Glucuronidated hyocholate and hyodeoxycholate are good substrates of human MRP3 (Zelcer et al. 2006). The glucuronidated bile salts are only formed in humans but not in mice (Zelcer et al. 2006); thus, further studies are needed to determine the role of MRP3 in bile acid efflux in humans. Physiological function of hepatic Mrp3 in excretion of bilirubin glucuronides from the liver into the circulation during cholestasis is demonstrated in *Abcc3*$^{-/-}$ mice. The *Abcc3*$^{-/-}$ mice are unable to excrete bilirubin glucuronides from the liver into the circulation during bile duct ligation, resulting in lower serum bilirubin glucuronide levels (Belinsky et al. 2005; Zelcer et al. 2006).

A series of papers have been demonstrated that MRP3 has a defense function and contributes to the excretion of toxic organic anions. Relevant pharmacological examples of MRP3 substrates include the glucuronide conjugates of acetaminophen (McGill and Jaeschke 2013), morphine (Hasegawa et al. 2009), and diclofenac (Scialis et al. 2015). Morphine is particularly an interesting case. Two glucuronide conjugates morphine 3-glucuronide and morphine 6-glucuronide are formed during its metabolism in humans. The two metabolites have differing pharmacologic properties. Morphine 3-glucuronide, the more abundant metabolite, has been shown to be antagonistic against the pharmacologic activities of morphine. Morphine 6-glucuronide, although less abundant, is pharmacologically active and is responsible for the majority of the therapeutic benefit of morphine. In addition, formation of morphine 6-glucuronide do not occur in mice. Zelcer et al. (2005) reported that relative to wild-type mice, *Abcc3*$^{-/-}$ mice showed low plasma levels of morphine 3-glucuronide and urinary excretion, whereas its levels in the liver and bile were increased following intraperitoneal administration of morphine. Associated with these pharmacokinetic differences, *Abcc3*$^{-/-}$ mice possessed low plasma concentration of morphine 6-glucuronide and weak antinociceptive potency compared with wild-type mice following intraperitoneal administration of morphine 6-glucuronide (Lickteig et al. 2007; Dzierlenga et al. 2015). Several reports (Dzierlenga et al. 2015; Lickteig et al. 2007; Canet et al. 2015) demonstrated that

nonalcoholic steatohepatitis upregulated expression of hepatic MRP3 and altered MRP2 location. Relative to control rats, morphine systemic exposure (*AUC*) decreased in nonalcoholic steatohepatitis rats to 74% of control, and morphine 3-glucuronide exposure increased to 150% of control. On the contrast, hepatic morphine 3-glucuronide concentration decreased to 37% of control. Urinary excretion of morphine 3-glucuronide showed a trend to increase in rats although no significance was obtained. As expected, nonalcoholic steatohepatitis rats exhibited a higher response to morphine 6-glucuronide in terms of area under the withdrawal latency curve, a measure of the time lapse between thermal stimulus and paw withdrawal (Dzierlenga et al. 2015). A clinical report (Ferslew et al. 2015) also showed that morphine glucuronide geometric mean C_{max} and $AUC^{0\text{-last}}$ was 52% and 58% higher, respectively, in patients with nonalcoholic steatohepatitis compared to healthy subjects following intravenous morphine administration. Severity of nonalcoholic steatohepatitis was associated with the increases in exposure of morphine glucuronide and levels of fasting serum bile salts (glycocholate, taurocholate, and total bile acid concentrations). The increased hepatic basolateral efflux of morphine glucuronide and bile acids is consistent with altered hepatic transport protein expression in patients with nonalcoholic steatohepatitis (Canet et al. 2015). Similarly, patients with nonalcoholic steatohepatitis also showed the increased serum and urinary levels of acetaminophen glucuronide, which is partly due to MRP3 induction or altered MRP2 localization (Canet et al. 2015). Methotrexate is also an MRP3 substrate. A report (Kitamura et al. 2008) showed that plasma concentrations of methotrexate after oral administration were significantly lower in *Abcc3*$^{-/-}$ mice than in wild-type mice, whose C_{max} was only 48% of wild-type mice. In consistence, total clearance and biliary clearance were 1.6-fold greater in *Abcc3*$^{-/-}$ mice than in wild-type mice. The intrinsic efflux clearance of methotrexate across the serosal membrane of the intestine in *Abcc3*$^{-/-}$ mice was significantly decreased to 24% of that in wild-type mice. On the contrast, the methotrexate concentration in the intestine of *Abcc3*$^{-/-}$ mice was 1.9-fold higher than in wild-type mice, suggesting that the lower intrinsic efflux clearance of methotrexate in *Abcc3*$^{-/-}$ mice is due to impaired efflux across serosal membrane of the intestine. Similarly, intestinal Mrp3 accounts for the serosal efflux of folic acid and leucovorin (Kitamura et al. 2010).

MRP3 may play an important role in protection against toxicity of some drugs. Scialis et al. (2015) reported that diclofenac acyl glucuronide concentrations in plasma of *Abcc3*$^{-/-}$ mice were 90% lower than in wild-type mice, indicating that Mrp3 mediates basolateral efflux of diclofenac acyl glucuronide. But no differences in biliary excretion of diclofenac acyl glucuronide between two strains were observed. Susceptibility to toxicity was also evaluated after a single high diclofenac dose. No signs of injury were detected in the livers and kidneys, but severe ulcers were found in the small intestines of the two strains. Furthermore, the intestinal injuries were consistently more severe in *Abcc3*$^{-/-}$ mice compared with wild-type mice, suggesting roles of intestinal Mrp3 in basolateral efflux of diclofenac acyl glucuronide. Uptake of glucuronide conjugates from intestinal lumen into enterocytes may be predominantly mediated by OATP2B1 in the human intestine

(Drozdzik et al. 2014) and by Oatp2b1 in mouse (Cheng et al. 2005), respectively. Then these glucuronide conjugates are pumped out of the cells via basolateral efflux or apical efflux. In *Abcc3*$^{-/-}$ mice, the basolateral efflux of diclofenac acyl glucuronide is attenuated, leading to higher accumulation of intracellular diclofenac acyl glucuronide within enterocytes and enhancing intestinal injury, but roles of diclofenac acyl glucuronide in development of intestinal injury is unclear. Other transporters such as Mrp2 and Mrp1 also play a role in protection against toxicity of diclofenac acyl glucuronide. In addition, Fernández-Barrena et al. (2012) reported that lack of Mrp3 expression markedly impaired liver growth in response to bile acids and after partial hepatectomy. It was found that lack of Mrp3 significantly reduced liver growth elicited by cholic acid feeding, farnesoid X receptor (FXR) activation, and portal serum levels of bile acids. Liver regeneration was significantly delayed in *Abcc3*$^{-/-}$ mice. Proliferation-related gene expression and peak DNA synthesis in *Abcc3*$^{-/-}$ mice occurred later than in wild-type mice, coinciding with a retarded elevation in intrahepatic bile acid levels.

*2.3.4 Multidrug Resistance Protein 4 (MRP4/*ABCC4*)*

2.3.4.1 General Properties of MRP4

MRP4/Mrp4, encoded by the *ABCC4/Abcc4* (*ABCC4* for human and *Abcc4* for animals) gene on chromosome 13q32.1, is the smallest of the MRP proteins with a length of 1325 amino acids, whose secondary structure resembles that of MRP5 (Borst et al. 2007). MRP4 is widely expressed in most tissues including the lung, kidney, bladder, gallbladder, small intestine, and tonsil. The highest expression of MRP4 occurs in the prostate, next to moderate expression in the lung, skeletal muscle, pancreas, spleen, thymus, testis, ovary, and small intestine. A unique feature of MRP4 is its dual localization in polarized cells. MRP4 may be located in apical or basolateral membrane of the targeted cells depending on cell and tissue specificity. For example, MRP4 is located in the basolateral membrane of the tubule acinar cells in the human prostate, hepatocytes, and pancreatic ductular epithelial cells. On the contrast, MRP4 is expressed in the apical membrane of the kidney proximal tubule brush border. In the brain, MRP4 is present in the basolateral membrane of the choroid plexus epithelium and in the luminal side of capillary endothelium. However, in primary cultured bovine brain microvessel endothelial cells, MRP4 is equally distributed in the apical and basolateral plasma membrane (Schinkel and Jonker 2012; Slot et al. 2011; Borst et al. 2007). With respect to enterocyte epithelium, MRP4 is localized to both the basolateral and apical membranes (Wen et al. 2015).

2.3.4.2 Pharmacological and Physiological Function of MRP4

MRP4 is first functionally identified as a transporter of antiviral agent adefovir and AMP analogue 9-(2-phosphonylmethoxyethyl) adenine (PMEA) using PMEA-resistant cell lines (Schuetz, et al. 1999). The PMEA-resistant cell lines are also cross-resistant to GMP analogue 9-(2-phosphonylmethoxyethyl) guanine (PMEG), azidothymidine (AZT), and 2′,3′-dideoxy-3′-thiacytidine (3TC). Importantly, the antiviral efficacy of PMEA, AZT, and 3TC is decreased substantially in the PMEA-selected cell lines. As a drug transporter, MRP4 possess broad substrates covering antiviral (adefovir, tenofovir, ganciclovir), antibiotic (cephalosporins), cardiovascular (loop diuretics, thiazides, and angiotensin II receptor antagonists), cytotoxic agents (methotrexate, 6-thioguanine, 6-mercaptopurine, and topotecan), and endogenous molecules. Phosphorylated metabolites of thiopurine analogues and most nucleoside-based antivirals are also MRP4 substrates. A unique characteristic of MRP4 is its remarkable ability to transport a range of endogenous molecules including cyclic nucleotides, ADP, eicosanoids, urate, conjugated steroid hormones, folate, bile acids, and glutathione (Slot 2007; Wen et al. 2015). These endogenous molecules are often required for a variety of physiologic processes, inferring that MRP4 may be involved in many physiological and pathophysiological processes via effluxing these cellular endogenous molecules.

MRP4 and Cancer

MRP4 is the main transporter for signaling molecules cAMP and PGE2, in turn, regulating cellular proliferation, differentiation, and apoptosis. The elevated expression of MRP4 has been detected in drug-naïve tumors including neuroblastoma, prostate cancer, pancreatic cancer, and AML, all in which MRP4 expression is associated with a worse prognosis (Kochel and Fulton 2015). Zhao et al. (2014) reported that MRP4 expression is required for cell proliferation and tumorigenesis of NSCLC. MRP4 is highly expressed in both lung cancer tissues and most lung cancer cell lines. Knock-down of MRP4 inhibited cell growth and increased the percentage of cells in G1 phase. In accordance, comparison of different AML subtypes showed that the highest level of MRP4 is expressed in the least differentiated subtypes (Guo et al. 2009). In the human leukemia cell line U937, blockage of MRP4 using shRNA or MRP4 inhibitor probenecid caused intracellular accumulation of cAMP and consequent leukemic maturation toward a more differentiated phenotype (Copsel et al. 2011). These findings are consistent with the apparent role for MRP4 and cAMP-mediated signaling in normal hematopoietic cell development, where MRP4 expression levels decrease during differentiation toward mature leukocytes (Oevermann et al. 2009). In leukemic stem cells, co-incubation with forskolin or MK571, either alone or in combination, induced CD38 expression, which co-incubation with both agents yielded significantly stronger inductive effect. Several studies have demonstrated roles of elevated intracellular cAMP in promoting morphological differentiation and decreasing proliferation in cultured tumor cell lines (Prasad et al. 2003; Sanchez et al. 2004). PDE4 inhibitor rolipram, MRPs inhibitor probenecid, or MRP4 knockdown using genetic silencing inhibited tumor growth in a mouse xenograft model (Copsel et al. 2014).

COX-2-PGE2 signaling pathway is highly activated in numerous cancers, especially lung and colon cancer. The elevated levels of COX-2 and PGE2 are indicators of a poor prognosis for cancers including breast cancer (Kochel et al. 2016; Glover et al. 2011; Kochel and Fulton 2015). Clinical studies have shown that adenoma development in familial adenomatous polyposis patients can be prevented by inhibiting prostaglandin production (Huynh et al. 2012; Chell et al. 2006). In neuroblastoma cell lines, adding PGE2 analogue (16,16-dimethyl PGE2) increased cell viability and proliferation in a dose-dependent manner and completely abrogated celecoxib-mediated cytotoxicity (Rasmuson et al. 2012). $Apc^{Min/+}$ mouse models have given direct evidence of prostaglandin-promoted tumor growth, with an increase in tumor incidence following PGE2 treatment (Greenhough et al. 2009). These results suggest that MRP4 also is involved in cancer development via regulating both cAMP and PGE2 (Kochel and Fulton 2015) and that MRP4 may be served as potential pharmacologic target for cancer therapy.

MRP4 and Aggregation

MRP4 is highly expressed in the membrane of dense δ-granules in platelet (Jedlitschky et al. 2004). In normal δ-granules, MRP4 can trap ADP and other cyclic nucleotides, where these signal molecules are secreted and induce platelet aggregation during platelet activation. A clinical report (Jedlitschky et al. 2010) showed that patients with a platelet δ-storage pool deficiency (δ-SPD)-like phenotype possessed the reduced platelet adenine nucleotide levels but normal serotonin levels. Immunoblotting analysis demonstrated that MRP4 expression in the platelets of the two patients with δ-SPD platelets was severely diminished. In platelets with "classic" δ-SPD (low adenine nucleotide and serotonin levels), total expression of MRP4 was similar to normal platelets, but localization of MRP4 was significantly changed. In classic δ-SPD, MRP4 seemed to be expressed in patches at the plasma membrane. Thus, defective expression of platelet MRP4 was associated with selective defect in adenine nucleotide storage. As a result, ADP-induced aggregation is impaired by MRP4 deficiency or inhibition, leading to decreased ADP accumulation. Moreover, $Abcc4^{-/-}$ mice also showed disruption of both hemostasis and thrombosis, with a prolonged bleeding time and delayed carotid occlusion. The defect in $Abcc4^{-/-}$ platelet activation was confirmed by P-selectin exposure and serotonin secretion, which showed decreased α- and dense-granule secretion by $Abcc4^{-/-}$ platelets, respectively. However, *Abcc4* knockdown did not affect the level of ADP in resting nor activating platelets but significantly decreased the amount of secreted cAMP from platelets without affecting total cAMP level, indicating the defective Mrp4 platelet function may be due to low accumulation of cAMP in the platelet cytosol (Decouture et al. 2015).

MRP4 transports aspirin into dense granules in platelet and contributes to aspirin resistance. It was reported that MRP4 expression in platelets from patients treated with aspirin was significantly higher than in platelets of healthy volunteers (Mattiello et al. 2011), inferring MRP4 induction by aspirin. Importantly, antiaggregation effects of aspirin from platelets were reduced (Mattiello et al. 2011). In consistence, Massimi et al. (2014) reported that platelets from healthy volunteers receiving

15-day aspirin treatment showed more evident MRP4 expression compared with 1-day treatment. In human megakaryoblastic cell lines, expressions of MRP4 mRNA and protein were significantly increased following 48 h exposure of aspirin (50 μM) (Massimi et al. 2014). These findings were reproduced in vitro using platelets derived from megakaryocytes in culture transfected with MRP4 siRNA or MRP inhibitor (Mattiello et al. 2011; Massimi et al. 2014). Platelet MRP4 induction by aspirin itself possibly may lead to the decline in the cellular aspirin concentration and the decrease in the inhibitory effects of platelets during continuous exposure. The connection between aspirin-induced MRP4 expression and decreased cell toxicity was demonstrated in HEK 293 cell lines, where expressions of MRP4 are well correlated with reduced numbers of 7-aminoactinomycin D-positive cells as well as cytosolic accumulation of aspirin (Massimi et al. 2015). Other nonsteroidal anti-inflammatory drugs (NSAIDs) such as celecoxib, diclofenac, and naproxen also strongly induced expression of MRP4 mRNA and protein in human megakaryoblastic cell lines. Platelets of osteoarthritis patient treated with NSAIDs also possessed a higher expression of MRP4 and an increase in ADP-induced platelet aggregation compared to the patient without NSAID treatment (Temperilli et al. 2016).

MRP4 and Pulmonary Hypertension

Cyclic nucleotides (cAMP and cGMP) control multiple cardiovascular processes including cardiac hypertrophy, cardiac contractility, myocardial fibrosis, endothelial barrier function, vascular smooth muscle cell proliferation, and vasodilation. The two signal molecules are MRP4 substrates. MRP4 is highly expressed in vascular smooth muscle cells. The expression of MRP4 in these cells may be upregulated under pathological conditions (Belleville-Rolland et al. 2016). Importantly, pulmonary arteries from patients with idiopathic pulmonary arterial hypertension showed upregulation of MRP4 expression, which was reproduced in wild-type mice exposed to hypoxia (Hara et al. 2011). In wild-type mice, hypoxia resulted in a marked increase in right ventricular systolic pressure and distal pulmonary artery remodeling. Coadministration of MK571 may reverse the alteration induced by hypoxia. But this alteration by hypoxia did not occur in *Abcc4*$^{-/-}$ mice. In human coronary artery smooth muscle cells, knockout MRP4 with a siRNA significantly increased intracellular and decreased extracellular levels of both cAMP and cGMP. In human and mouse pulmonary artery smooth muscle cell, MRP4 silencing increased intracellular/extracellular ratios of both cAMP and cGMP. Additionally, coadministration of PDE inhibitor sildenafil further enhanced the changes in cyclic nucleotide levels by MRP4 silencing (Hara et al. 2011). In vivo, silencing Mrp4 using intratracheal delivery of aerosolized adeno-associated virus 1 to rats dose-dependently decreased monocrotaline-induced right ventricular systolic pressure and hypertrophy but also significantly reduced both in distal pulmonary arteries remodeling and expression of atrial natriuretic factor (Claude et al. 2015). These findings support a therapeutic potential for downregulation of MRP4 for the treatment of pulmonary artery hypertension.

MRP4 and Drug Toxicity/Efficacy

MRP4 acts as an endogenous regulator of intracellular cyclic nucleotide levels, and some drugs exhibit their pharmacological or toxic effect via affecting MRP4 expression and function. For example, expression of intestinal MRP4 is associated with drug-induced diarrhea. Diarrhea is a common side effect of ~7% of all adverse drug reactions and results from impaired fluid secretion in the intestine. Fluid homeostasis and secretion in the intestine are mainly controlled by cystic fibrosis transmembrane conductance regulator (CFTR). The physical and functional coupling of MRP4 with CFTR directly infers that MRP4 may affect CFTR-induced ion secretion via restricting and modulating compartmentalization of cAMP signaling. In line, MRP4 inhibitor MK571 may potentiate adenosine-stimulated CFTR-mediated chloride currents (Li et al. 2007).

Irinotecan-induced diarrhea may partly be attributed to intestinal MRP4. It was reported that *Abcc4*$^{-/-}$mice were resistant to irinotecan-induced fluid secretion (Moon et al. 2015). In wild-type mice, irinotecan treatment significantly increased fluid secretion in ileal loops in a dose-dependent manner; this effect did not occur in *Abcc4*$^{-/-}$ mice. The irinotecan-induced fluid secretion in wild-type mice may be completely inhibited by CFTR inhibitor CFTRinh-172 or MK571. In *Abcc4*$^{-/-}$ mice, forskolin, which increases intracellular cAMP, did induce intestinal fluid secretion but neither irinotecan nor MK571 did. In HT29-CL19A cells, both irinotecan and MK571 significantly evoked cAMP levels, and MRP4-mediated transport of [^{3}H]cAMP was inhibited by irinotecan. All these results indicate that CFTR-MRP4- complexes play an important role in the pathogenesis of drug-induced diarrhea. On the contrast, patients suffered from irritable bowel syndrome with constipation showed decreased expression of intestinal MRP4 (Harrington et al. 2014). Linaclotide, a potent and selective agonist of guanylate cyclase-C, is used for the treatment of patients suffered from irritable bowel syndrome with constipation and chronic constipation via enhancing intestinal secretion and transit. Linaclotide (Tchernychev et al. 2015) was reported to induce a concentration-dependent increase in transepithelial ion current across rat colonic mucosa via activating guanylate cyclase-C/cGMP pathway, which was also involved in MRP4. MRP4 inhibitor MK571 potentiated linaclotide-induced electrolyte secretion and augmented linaclotide-stimulated intracellular cGMP accumulation. Linaclotide stimulated cGMP secretion from the apical and basolateral membranes of colonic epithelium. MRP4 inhibition blocked cGMP efflux from the apical membrane. These data reveal a novel mechanism that functionally couples guanylate cyclase-C-induced luminal electrolyte transport and cGMP secretion to spatially restricted, compartmentalized regulation by MRP4 at the apical membrane of intestinal epithelium.

HIV protease inhibitors such as amprenavir, indinavir, saquinavir, ritonavir, and nelfinavir are MRP4 substrates, but in Saos-2 and HEK293 cells expressing MRP4, it was reported that only nelfinavir is good substrate and inhibitor of MRP4. Nelfinavir strongly increased intracellular PMEA levels and reduced MRP4-mediated resistance to methotrexate, leading to significant increases in PMEA cytotoxicity and methotrexate cytotoxicity, which were reproduced in cell lines

from *Abcc4*$^{-/-}$ mice. Cell lines from *Abcc4*$^{-/-}$ mice showed more sensitivity to nelfinavir than wild-type mice. On the contrast, the enhanced MRP4 levels reduced nelfinavir accumulation and cytotoxicity. These data demonstrate that nelfinavir increases the toxicity substrates (such as adefovir and methotrexate, respectively) via reducing their export (Fukuda et al. 2013). The inhibition of MRP4-mediated drug export has the potential to alter metabolism and distribution of drugs, affecting their efficacy and toxicity. Tenofovir disoproxil fumarate is an oral prodrug of tenofovir. Clinical reports showed that some patients treated with tenofovir disoproxil fumarate discontinued due to acute kidney injury. A report showed that levels of [^{3}H]tenofovir in the kidney of *Abcc4*$^{-/-}$ mice were higher than wild-type mice, but plasma concentrations [^{3}H]tenofovir in the two strain mice were comparable (Imaoka et al. 2007). *Abcc4*$^{-/-}$ mice demonstrated that tenofovir disoproxil fumarate significantly increased mtDNA abundance of proximal tubules. Notably, tenofovir disoproxil fumarate-treated wild-type mice and untreated *Abcc4*$^{-/-}$ mice also demonstrated a trend of increased mtDNA abundance compared to untreated wild-type mice, although no significance was obtained. Analysis of fine structure of kidney tissues in tubular epithelium showed that *Abcc4*$^{-/-}$ mice treated with tenofovir disoproxil fumarate exhibited increased number of mitochondria, with irregular mitochondrial shape, and sparse, fragmented cristae, inferring that *Abcc4*$^{-/-}$ mice are more susceptible to tenofovir mitochondrial toxicity (Kohler et al. 2011).

Thiopurines are effective immunosuppressants and anticancer agents, but intracellular accumulation of their active metabolites (6-thioguanine nucleotides) causes serious hematopoietic toxicity. Krishnamurthy et al. (2008) reported that wild-type mice and *Abcc4*$^{-/-}$ mice intraperitoneally received 6-mercaptopurine with 50, 100, or 150 mg/kg daily for 15 days. All *Abcc4*$^{-/-}$ mice died by day 13 regardless of dosages, whereas >75% of the wild-type mice survived at day 15. Following 5-day 6-mercaptopurine treatment (100 mg/kg daily), the granulocyte and monocyte-macrophage progenitors were reduced 71% and 74%, respectively, in *Abcc4*$^{-/-}$ mice compared with untreated controls, but these decreases in in wild-type mice were less than 20%. Cytotoxicity of 6-mercaptopurine toward erythroid progenitors was greater in *Abcc4*$^{-/-}$ mice than in wild-type mice. Bone marrow erythroid progenitor reduction in 6-mercaptopurine-treated mice was paralleled by reduction in blood hemoglobin concentration. In consistence, *Abcc4* $^{-/-}$ bone marrow cell showed increases in 6-thioguanine nucleotides. These findings are consistent with the anemia and dramatically reduced erythrocytes observed in patients experiencing thiopurine toxicity. MRP4 SNP (*rs3765534*, *c.2269G > A*) was identified in the Japanese population whose weighed average of all alleles was >18.7% allele frequency. In vitro studies showed that total levels of MRP4 protein in HapMap lymphocyte cell lines or HEK293 cells expressing MRP4 variant were similar compared with wild-type MRP4 allele, but the MRP4 variant less MRP4 membrane localization and the cells expressing MRP4 variant were much more susceptible to 6-mercaptopurine toxicity compared with wild-type MRP4 allele (Krishnamurthy et al. 2008). This frequent, less functional, MRP4 allele may account for enhanced thiopurine sensitivity in some Japanese (Krishnamurthy

et al. 2008). Another MRP4 variant (*rs11568658*), a low frequency (approx. 2%), was reported to be associated with decreased neutrophil counts following valganciclovir (Billat et al. 2016). Cells expressing MRP4 variant (*rs11568658*) demonstrated significantly higher accumulation of valganciclovir. The efflux process from the cell was almost abolished, inferring complete loss of transport activity (Billat et al. 2016). Cytarabine-induced hematological toxicity was also reported to be associated with MRP4 expression. Plasma concentration of cytarabine in *Abcc4*$^{-/-}$ mice following after intraperitoneal injection of the drug was comparable to wild-type, cytarabine-induced significant hematopoietic toxicity; the neutrophil counts were significantly decreased to 30% in untreated *Abcc4*$^{-/-}$ mice. Colony-forming assays further demonstrated that myeloid progenitors from *Abcc4*$^{-/-}$ mice was more sensitive to cytarabine (Drenberg et al. 2016).

2.3.5 Other Multidrug Resistance Proteins

2.3.5.1 Multidrug Resistance Protein 5 (MRP5)

Like MRP3 and MRP4, human MRP5 (gene symbol *ABCC5*) was first identified as homologues of MRP1 by database screening of expressed sequence tags (Kool et al. 1997). MRP5 shares only a 38% identity with MRP1. Main function of MRP5 is to transport cyclic nucleotides and their nucleotide analogues. Therefore, MRP5 (or MRP4, MRP8) is sometimes referred to as a "cyclic nucleotide efflux pump." MRP5 and MRP4 transport cAMP and cGMP and affect the intracellular transduction of these mediators. In addition, the two MRPs also export monophosphate nucleotide analogues, leading to resistance to nucleotide drugs (Fig. 2.5). Although MRP4, MRP5, and MRP8 transport both cAMP and cGMP, their affinities to cGMP and cAMP are greatly different. MRP5 is a high-affinity transporter for cGMP and low-affinity transporter of cAMP. Whereas MRP8 mediates transports of both cAMP and cGMP with moderate to low affinity (Chen and Tiwari 2011). These results infer that contribution of MRP5 to cGMP export is larger than other two MRPs. Interestingly, cGMP transport is highly sensitive to inhibitors of cGMP phosphodiesterase, such as zaprinast, trequinsin, and sildenafil (Jedlitschky et al. 2000).

MRP5 mRNA is expressed in most normal tissues at low levels, with maximum expression in the skeletal muscle, heart, brain, and cornea and only low levels in the liver (Schinkel and Jonker 2012; Slot et al. 2011; Borst et al. 2007). In polarized cells, MRP5 is preferentially located in the basolateral membrane, but MRP5 is expressed in the luminal side of brain capillary endothelial cells, pyramidal neurons, and subcortical white matter astrocytes. Importantly, as gestational age increases, the levels of MRP5 mRNA decrease significantly. In the preterm placentas, the majority of MRP5 is located in the basal membrane of the syncytiotrophoblasts. But in the term placentas, MRP5 is also located in the apical membrane of the syncytiotrophoblast, in addition to the basal membrane (Meyer et al. 2005).

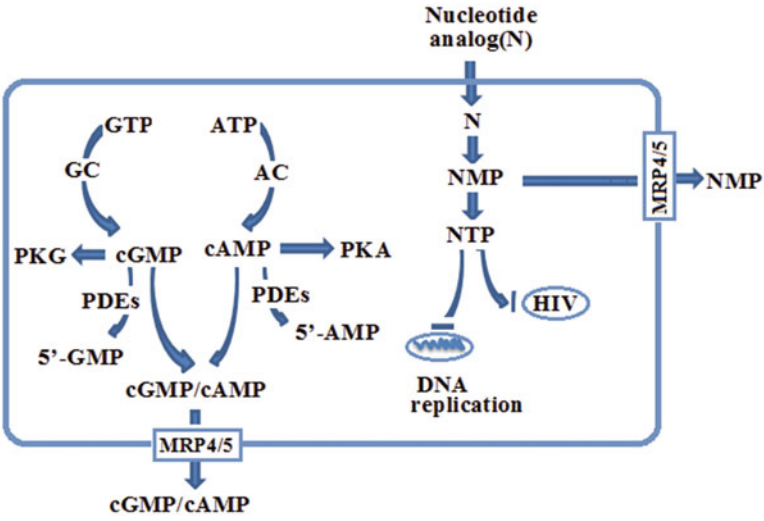

Fig. 2.5 Roles of MRP4 and MRP5 in cyclic nucleotide (cAMP/cGMP) signals and in resistance to nucleotide analogues (N). Overexpression of MRP4 or MRP5 can lower the intracellular concentrations of cAMP or cGMP, in turn, affecting the intracellular transduction of these mediators. Furthermore, monophosphate nucleotide analogues are exported from targeted cells by MRP4 and MRP5, lowering intracellular accumulation of monophosphate nucleotide analogues and impairing ability to inhibit virus replication or cell proliferation. Symbol: *HIV* HIV reverse virus, *N* nucleotide, *NMP* monophosphate nucleosides, *NTP* triphosphate nucleosides, *PK* protein kinase, *PDEs* phosphodiesterases, *AC* adenyl cyclase, and *GC* guanylyl cyclase

MRP5 functions as an export pump for cyclic nucleotides, especially cGMP, inferring that MRP5 is implicated in physiological processes via regulating cGMP levels. cGMP is synthesized in an NO-dependent manner and degraded by phosphodiesterases, particularly PDE5 or efflux via MRP5. In pituitary cells, MRP5 acts as a selective transporter of cGMP. Under resting states, the majority of cAMP exists within normal and immortalized pituitary cells, whereas the majority of cGMP exists extracellularly. It was found that growth hormone-releasing hormone (GHRH), corticotropin-releasing factor, and forskolin induced cAMP accumulation in both cytosol and extracellular medium but only increased cGMP levels in extracellular medium. Perifused pituitary cell experiment demonstrated that probenecid (1 mm) completely abolished GHRH-induced cGMP efflux although GHRH-induced cAMP efflux was also dramatically inhibited by probenecid. In consistence, knockdown of MRP5 using siRNA significantly attenuated cGMP release in CH3 cells without affecting cAMP release (Andric et al. 2006).

Both MRP5 mRNA and protein were detected in human heart, showing less MRP5 mRNA auricular samples than the ventricular samples. MRP5 were also expressed in auricular cardiomyocytes and ventricular cardiomyocytes. MRP5 expression in human heart is important, because several features of cGMP as a second messenger of nitric oxide (NO) have emerged in the heart, not only in the regulation of the vascular smooth muscle tone but also in the regulation of cardiac

contractility. Interestingly, expression of MRP5 in nonischemic cardiomyopathy and ischemic cardiomyopathy was significantly higher than that in the normal heart. This upregulation could be related to the ischemic preconditioning with enhanced tissue cGMP levels demanding enhanced cGMP elimination (Dazert et al. 2003).

MRP5 expressed in gastrointestinal smooth muscle also regulates gastrointestinal smooth muscle tone via exporting cGMP. It was found that expressions of MRP5 (proteins and mRNA) in muscle cells from the fundus were significantly higher than those in muscle cells from the antrum. In presences of PDE inhibitor isobutylmethylxanthine, NO donor *S*-nitrosoglutathione concentration-dependently increased extracellular levels of cGMP in both the fundus and antrum, but the maximum increase in the fundus was significantly higher than that in the antrum. Probenecid, depletion of ATP, or knockdown of MRP5 using siRNA blocked increases in extracellular cGMP by S-nitrosoglutathione. Moreover, activity of protein kinase G and muscle relaxation induced by S-nitrosoglutathione were also significantly lower in muscle cells from the fundus than that in the antrum. These results demonstrate that expression of MRP5 in muscle cells negatively correlates with tonic phenotype of muscle (Al-Shboul et al. 2013).

Mouse fibroblasts showed that suppression of Mrp5 using siRNA effectively reduced hyaluronan export. The hyaluronan export was also inhibited by MRP5 inhibitors, demonstrating important roles of MRP5 in hyaluronan efflux from fibroblasts. cGMP is a physiological substrate of MRP5 and the increase in the cGMP levels inhibited hyaluronan export. These results indicate that cGMP may be a physiological regulator of hyaluronan export at the level of the export MRP5 (Schulz et al. 2007). CFTR is one of the closest relatives to MRP5. Upregulation of the CFTR mRNA in *Abcc5* deficient mice was considered to compensate MRP5 for hyaluronan export (Schulz et al. 2010). MRP5 is present in BBB, neurons, and glia. *Abcc5*$^{-/-}$ mouse experiment demonstrated that efflux of glutamate conjugates and analogues is mediated by Mrp5, leading to higher accumulation of endogenous glutamate conjugates in several tissues, particularly in the brain, compared with wild-type mice. MRP5 also transports exogenous glutamate analogues including the classic excitotoxic neurotoxins (kainic acid and domoic acid) and the anticancer drug (methotrexate). Glutamate conjugates and its analogues are of physiological relevance, indicating that MRP5 may be involved in some physiological process via altering glutamate signaling or disposition of glutamate conjugate and analogues (Jansen et al. 2015).

MRP5 also mediates transport of other organic anions, such as S-(2, 4-dinitrophenyl) glutathione (DNP-SG), GSH, acyclovir, and adefovir. In vitro studies demonstrated 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, and 5-fluorouracil), and doxorubicin, and to antifolate drugs (Schinkel and Jonker 2012; Slot et al. 2011; Borst et al. 2007). Nevertheless, elevated levels of MRP5 mRNA were observed in lung cancer samples obtained following long-term treatment of patients with cisplatin (Oguri et al. 2000). Several studies have demonstrated

the expression of MRP5 prognostic roles in cancer. A study on 40 patients with metastatic colorectal cancer showed that $MRP5^{+}$ patients had a significantly shorter progression-free survival compared to patients, whose miss circulating tumor cells were negative for MRP5 expression (Gazzaniga et al. 2010). In patients with glioblastoma multiforme, patients with MRP5 index >11% exhibited significantly worse survival compared to those with MRP5 index<=11%. There was a significant increase in progression-free survival for patients with a MRP5 index lower than 11% (Alexiou et al. 2012). Further investigation is mandated to understand the role of MRP5 in mediating clinical resistance to antiretroviral, cancer chemotherapy, and cancer development.

2.3.5.2 Multidrug Resistance Protein 6 (MRP6/ABCC6)

MRP6, a 190 kDa protein coded by *ABCC6* gene, was first identified in epirubicin-resistant human leukemic cells (Slot et al. 2011; Chen and Tiwari 2011). The mouse and rat Mrp6 orthologs show greater than 78% amino acid identity with human MRP6. Human MRP6 also shares 45% amino acid identity with MRP1. The highest levels of MRP6 mRNA and protein expression are detected in the liver and kidney, although low levels have been detected in most other tissues, including the skin and retina. Like MRP1 and MRP3, MRP6, located in the basolateral membrane of polarized endothelial and epithelial cells, is also an organic anion transporter, but glucuronide conjugates (e.g., estradiol glucuronide) are not its substrates. Recessive mutations in *ABCC6* gene are responsible for a rare human genetic disorder known as pseudoxanthoma elasticum (PXE). In vitro, MRP6 can mediate transport of GSH conjugates LTC4 and DNP-SG but not glucuronide conjugates. Cells transfected with MRP6 also show low levels of resistance to a series of anticancer drugs such as etoposide, teniposide, doxorubicin, daunorubicin, actinomycin D, and cisplatin. No evidences support the role of MRP6 in clinical multidrug resistance.

2.3.5.3 Multidrug Resistance Protein 7 (MRP7/ABCC10)

MRP7 (gene symbol *ABCC10*), a protein consisting of 1492 amino acids, was firstly identified and nominated through complementary DNA library search (Slot et al. 2011; Chen and Tiwari 2011). Compared with the other long MRPs, MRP7 shares its low sequence identity to other MRP members and lacks N-linked glycosylation in the N-terminus. MRP7 is expressed in the pancreas, colon, skin, and testes. MRP7 transports physiological substrates including glucuronide conjugates (such as $E_2$17βG) and GSH conjugates (such as LTC4). MRP7 can also confer resistance to antimitotic agents (vincristine and docetaxel) and certain nucleoside analogues without resistance to anthracyclines (e.g., doxorubicin). Contribution of MRP7 to multidrug resistance has been confirmed by $Abcc10^{-/-}$ mice both in vitro and in vivo (Hopper-Borge et al. 2011). Mouse embryo fibroblast from $Abcc10^{-/-}$

mice showed hypersensitivity to docetaxel, paclitaxel, vincristine, and Ara-C compared with wild-type mice. The *Abcc10*$^{-/-}$ mice showed hypersensitivity to paclitaxel (the minimal toxic dose 32 mg/kg in *Abcc10*$^{-/-}$ mice versus 150 mg/kg in wild-type mice). Markedly impaired bone marrow, spleen, and thymus as well as decrease in blood cell counts were observed in *Abcc10*$^{-/-}$ mice, indicating that Mrp7 affords protection against drug-induced bone marrow toxicity and immunity impairment. Some small molecule inhibitors of tyrosine kinases (such as nilotinib, imatinib, erlotinib, lapatinib, and tandutinib) and specific EphB4 receptor inhibitor (NVP-BHG712) can enhance efficacy of paclitaxel via affecting MRP7 function. In xenograft model, coadministration of nilotinib, masitinib, or NVP-BHG712 significantly decreased the HEK293/MRP7 tumor size, weight, and its growth over a period of 18 days (Tiwari et al. 2013; Kathawala et al. 2014; Kathawala et al. 2015). The pharmacokinetic data showed that coadministration of masitinib or NVP-BHG712 significantly increased the intratumoral concentration of paclitaxel without affecting plasma concentration of paclitaxel. In vitro studies showed that masitinib or NVP-BHG712 significantly enhanced the sensitivity of HEK293/MRP7 cells to paclitaxel and the cellular accumulation of paclitaxel without affecting the expression levels of MRP7 (Kathawala et al. 2014; Kathawala et al. 2015). PDE5 inhibitors sildenafil and vardenafil also enhanced the sensitivity of HEK293/MRP7 cells to paclitaxel, docetaxel, and vinblastine without affecting the protein expression and translocation of MRP7 (Chen et al. 2012).

2.3.5.4 Multidrug Resistance Protein 8 (MRP8/ABCC11)

Human MRP8, a protein consisting of 1382 amino acids coded by *ABCC11* gene, was first identified in breast cancer using a gene prediction program and EST database mining (Bera et al. 2001; Chen and Tiwari 2011). Like MRP4 and MRP5, MRP8 is a short MRP and localized to apical membranes in stably transfected polarized epithelial cells. Unlike other MRPs, no orthologous genes have been found in mammals except for primates. The MRP8 protein is expressed in axons of neurons, in the human central and peripheral nervous systems. It mediates the efflux of neuromodulatory steroids such as dehydroepiandrosterone 3-sulfate. Data from membrane vesicles from LLC-PK1 cells expressing MRP8 demonstrated that MRP8 can transport a wide range of compounds including cyclic nucleotides (such as cGMP and cAMP), bile acids, sulfated and glucuronidated steroids, or other conjugated organic anions such as LTC4 (Chen et al. 2005). Cell lines expressing MRP8 can confer resistance to a range of clinically relevant nucleotide analogues such as anticancer fluoropyrimidines (5′-fluorouracil, 5-′-fluoro-2′-deoxyuridine, and 5′-fluoro-5′-deoxyuridine), the antihuman immunodeficiency virus agents (2′,3′-dideoxycytidine), and the anti-hepatitis B agent (PMEA) or methotrexate (Guo et al. 2003). High expression of MRP8 is demonstrated in some drug-resistant cells. Pemetrexed was approved for the treatment of malignant pleural mesothelioma and NSCLC. The pemetrexed-resistant lung cancer cell lines

demonstrated higher expression of MRP8 compared with the parental cells. The resistant cells showed cross-resistance to methotrexate (Uemura et al. 2010). Similarly, 5-fluorouracil-resistant small-cell lung cancer cell line PC-6/FU23-26 also exhibited higher expression of MRP8 compared with parent PC-6 cells and low cytotoxicity to 5-FU, accompanied by low intracellular 5-fluoro-2′-deoxyuridine 5′-monophosphate accumulation. The PC-6/FU23-26 cells were also resistant to methotrexate. The resistance to methotrexate and 5-FU was attenuated by *ABCC11* siRNA (Oguri et al. 2007).

Physiologically MRP8 is associated with axillary odor and secretion of cerumen (earwax). One SNP (*rs17822931*; *c.538G > A*, p.Gly180Arg) of MRP8 was reported to determine the human earwax type. Earwax is a secretory product of the ceruminous apocrine glands, which can be classified into two phenotypes in humans, wet (sticky) and dry. The AA genotype gives the dry phenotype, whereas both GA and GG genotypes give the wet phenotype. The dry type is mostly common in the Asian population, especially in Korean, Japanese, and Chinese, whereas the wet type is a dominant phenotype for many Africans and Caucasians (Toyoda and Ishikawa 2010). An association between breast cancer risk and wet earwax was observed in Japanese women (Ota et al. 2010). The odds ratio for the genotypes (G/G + G/A) to develop breast cancer was estimated to be 1.63, suggesting that the G allele in *ABCC11* is associated with breast cancer risk. However, this association for allele and genotype frequencies of the c.538G > A variant in ABCB11 with breast cancer risk was not observed in Caucasian (Lang et al. 2011; Beesley et al. 2011), inferring that the effect of MRP8 genotypes on breast cancer is likely to be more complex. Several reports have demonstrated that expression of MRP8 is often associated with poor clinical outcome and that expression of MRP8 may be served as a predictive marker for treatment outcome in some cancers. In AML patients, high expression of MRP8 was reported to be associated with a low probability of overall survival assessed over 4 years. Higher levels of MRP8 transcripts were observed in the less differentiated French-American-British subtypes M0 and M1 compared with the M2 subtype. LLC-PK1 cells expressing MRP8 were also resistant to Ara-C due to efflux of Ara-C metabolites (Guo et al. 2009).

Expression of MRP8 is associated with poor output in breast cancer risk. Yamada et al. (2013) compared expression of MRP1, MRP8, and BCRP in subtypes of the 281 breast cancer samples (luminal A, luminal B, HER2, triple-negative). The triple-negative tumors were further subdivided into two groups, core-basal and five-negative. The results showed that although MRP1, MRP8, and BCRP were highly expressed in aggressive breast cancer subtypes, tumor MRP8 expression was associated with poor prognosis. Importantly, patients with MRP8-positive tumors had significantly worse prognoses than did patients with MRP8-negative tumors, except for the luminal A subtype, which is known to have a better prognosis than the other subtypes. In agreement, patients with high tumor expression of MRP8 had worse outcomes, particularly among the HER2-enriched and core-basal subtypes. Further studies are needed to provide insight into whether MRP8 plays any role in clinical multidrug resistance.

2.4 Breast Cancer Resistance Protein (BCRP/ABCG2)

2.4.1 General Properties and Distribution of BCRP

Human breast cancer resistance protein (BCRP) also refers to as placenta-specific ABC transporter (ABCP) or mitoxantrone resistance-associated protein (MXR). Human BCRP and animal Bcrp are coded by *ABCG2* and *Abcg2*, respectively. BCRP belongs to a half-sized ABC transporter, which BCRP functions as a homodimer. BCRP was first identified in a highly doxorubicin-resistant MCF-7 breast cancer cell line (MCF-7/AdrVp). Human BCRP gene encodes a 655 amino acid protein. The murine homologue, Bcrp, is highly overexpressed in mouse fibroblasts selected for resistance to doxorubicin, mitoxantrone, or topotecan. *Abcg2* encodes a 657 amino acid protein with 81% identity to human BCRP. BCRP is prominently expressed in the placental syncytiotrophoblast plasma membrane facing the maternal bloodstream, canalicular membrane of the hepatocytes, and luminal membrane of villous epithelial cells in the small and large intestine, locations shared with P-GP (Kusuhara and Sugiyama 2007; Mao and Unadkat 2014). BCRP is also expressed in the apical side of the part of the ducts and lobules in the breast, and in the venous and capillary endothelial cells of practically all tissues analyzed, but not in arterial endothelium. The specific distribution profile of BCRP is closely related to the physiological role of BCRP (Kusuhara and Sugiyama 2007; Mao and Unadkat 2014). Importantly, BCRP is also present at high levels in primitive hematopoietic stem cells and other stem cells including cancer stem, whose expression may be used as a marker for stem cells (Fatima et al. 2012). However, little or no expression of Bcrp is detected in more differentiated hematopoietic lineages, with the exception of erythroid precursor cells and natural killer lymphocytes (Scharenberg et al. 2002).

2.4.2 Substrates and Inhibitors of BCRP

Cell line-resistant mitoxantrone, topotecan, doxorubicin, and SN-38 all overexpress BCRP, inferring that these drugs are BCRP substrates. But, vincristine, paclitaxel, and cisplatin are not BCRP substrates. A series of BCRP substrates have been identified directly by cellular or vesicular transport assays or indirectly by substrate-stimulated ATPase activity or cytotoxicity assays, comprising a broad spectrum of anticancer drugs, sulfate and glucuronide conjugates of sterols and xenobiotics, natural compounds and toxins, fluorescent dyes, photosensitizers (such as pheophorbide A, protoporphyrin IX, and related compounds), and some antibiotics. Other drugs such as prazosin, glyburide, cimetidine, sulfasalazine, rosuvastatin, AZT, lamivudine, vitamin B2, and vitamin K3 and some chemical carcinogens (such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) are also BCRP substrates. BCRP also transports conjugated organic anions including

endogenous substances (such as estrone 3-sulfate, dehydroepiandrosterone sulfate, and $E_2$17βG). In general, sulfated conjugates seem to be better BCRP substrates than glutathione and glucuronide conjugates. In addition, nucleotides and their monophosphates such as AZT 5′-monophosphate are also BCRP substrates. Estrone 3-sulfate and dehydroepiandrosterone sulfate are considered to be the potential physiological substrates of BCRP. BCRP is also a high-capacity urate exporter, and its dysfunction may become a major cause of gout (Matsuo et al. 2011). It should be noted that BCRP substrates are often substantially overlapped with other ABC transporters such as P-GP and MRPs.

Interestingly, BCRP protein acquired during the course of drug selection are responsible for differential drug efflux and sensitivity. For example, a report showed (Honjo et al. 2001) that nine cell lines overexpressing BCRP proteins all transported mitoxantrone, topotecan, and SN-38, but only two (MCF-7 AdVp3000 and S1-M1-80) transported rhodamine 123. Sequence analysis revealed that BCRP proteins expressed in MCF-7/AdVp3000 cell lines and S1-M1-80 cell lines are mutant BCRP, with a threonine (R482T) and glycine (R482G) at position 482, respectively. BCRP protein expressed in other cell lines is wild-type (R482) protein with an arginine at position 482. Both MCF-7 AdVp3000 cells (R482T) and S1-M1-80 (R482G) cells show more resistant to doxorubicin than MCF-7 MX100 (R482) does. HeLa cells expressing wild-type or the mutant BCRP all show the reduced cellular mitoxantrone accumulations, but only cells transfected with R482T or R482G show low rhodamine 123 and doxorubicin accumulations. However, wild-type BCRP and the two mutants can all bind directly to IAARh123, indicating that the inability of the wild-type BCRP to transport rhodamine 123 may not occur at the initial binding step (Alqawi et al. 2004). Similarly, HEK-293 cells expressing wild-type or mutant BCRP also confirm that both wild-type and the two mutants are able to efflux mitoxantrone, topotecan, SN-38, Hoechst 33342, and BODIPY-prazosin. But only R482G and R482T mutants have higher affinity with doxorubicin, daunorubicin, epirubicin, bisantrene, rhodamine 123, and LysoTracker Green (Robey et al. 2003). Only wild-type BCRP efficiently transports methotrexate, methotrexate diglutamate, methotrexate triglutamate, folic acid, and $E_2$17βG (Volk and Schneider 2003; Chen et al. 2003). However, Shafran et al. (2005) reported that cells expressing mutant BCRP (R482G) were 6-, 23-, and > 521-fold more resistant to methotrexate, GW1843, and Tomudex, respectively, compared with wild-type BCRP cells. Interestingly, BCRP proteins were resistant to antifolates in a dependent time manner. HEK293 cells overexpressing the mutant G482 BCRP displayed 120-, 1000-, and > 6250-fold resistance to methotrexate, GW1843, and Tomudex following 4 h drug exposure. In consistence, following 4 h incubation of 1 μmol/L [^{3}H]methotrexate, cells expressing wild-type or mutant BCRP showed a twofold decrease in [^{3}H] methotrexate accumulation, relative to parental cells, which may be associated with high-level resistance to methotrexate. But, cells expressing mutant or wild-type BCRP lost almost all their antifolate resistance following 72 h drug exposure. It was contrast to hydrophilic antifolates that HEK293 cells expressing mutant BCRP were more resistant to lipophilic antifolates compared with wild-type BCRP (Bram et al. 2006). Table 2.6 lists the selected substrate drugs of wild-type BCRP.

Table 2.6 Summary of wild-type BCRP substrates

Anthracenes	Mitoxantrone, bisantrene, aza-anthrapyrazole
Camptothecin derivatives	Topotecan, SN-38, irinotecan, diflomotecan
Polyglutamates	Methotrexate, methotrexate diglutamate, methotrexate triglutamate
Nucleoside analogues	Zidovudine, zidovudine 5′-monophosphate, lamivudine
Photosensitizers	Pheophorbide a, protoporphyrin IX, hematoporphyrin
Others	Prazosin, indolocarbazole, flavopiridol, canertinib, imatinib mesylate, gefitinib, nilotinib, glyburide, cimetidine, sulfasalazine nitrofurantoin, rosuvastatin, pantoprazole

Elacridar, a highly efficient P-GP inhibitor, is also an effective BCRP/Bcrp1 inhibitor. Other P-GP inhibitors, such as reserpine, cyclosporin A, tariquidar, and valspodar, also inhibit BCRP activity. Fumitremorgin C (FTC), a tremorgenic mycotoxin produced by the fungus *Aspergillus fumigatus*, specifically inhibits BCRP activity with less effect on P-GP and MRP1 activities. Unfortunately, clinical development of FTC is not possible due to its neurotoxicity. A series of FTC analogues have been generated. Two most potent analogues (Ko132 and Ko134) have comparable or greater activity than FTC. Ko134 has low cytotoxicity in vitro, and it can be given at high oral dosages to mice. FTC analogues of this type may thus be useful for development of clinical BCRP inhibitors.

Several tyrosine kinase inhibitors (TKIs), such as gefitinib, imatinib mesylate, nilotinib, erlotinib, lapatinib, and sunitinib, have been shown to inhibit BCRP-mediated drug resistance. These TKIs themselves are BCRP substrates, indicating it is possible that these compounds may act as competitive BCRP inhibitors.

Other potent BCRP inhibitors include novobiocin, UCN-01 (a cyclin-dependent kinase inhibitor), and some flavonoids (such as silymarin, hesperetin, quercetin, and daidzein). Interestingly, novobiocin only inhibits wild-type BCRP activity without affecting mutant BCRP activity (Robey et al. 2003). HIV protease inhibitors ritonavir, saquinavir, nelfinavir, and lopinavir can effectively inhibit wild-type BCRP activity but with less effect on R482T/R482G mutants. But, the four HIV protease inhibitors are not BCRP substrates (Gupta et al. 2004).

2.4.3 Pharmacological and Physiological Functions of BCRP

2.4.3.1 Roles of BCRP in Disposition and Toxicity/Efficacy of Drugs

The tissue distribution of BCRP shows extensive overlapping with that of P-GP, suggesting a pharmacological and toxicological protective role of BCRP, similarly to that of P-GP. The functional importance of BCRP in normal tissues has been identified using *Abcg2* $^{-/-}$ mice or coadministration of BCRP inhibitors. For example, oral coadministration of elacridar (P-GP/BCRP inhibitor) induced a sixfold

increase in the plasma exposure of topotecan following oral dose to *Mdr1a/1b*$^{-/-}$ mice compared with vehicle-treated mice, accompanied by decreases in hepatobiliary excretion of topotecan and increases in intestinal uptake (Jonker et al. 2000). In pregnant *Mdr1a/1b*$^{-/-}$ mice, elacridar induced over twofold increase in fetal distribution of topotecan compared with vehicle-treated mice (Jonker et al. 2000). Similarly, *Abcg2*$^{-/-}$ mice showed sixfold increase in oral availability of topotecan and twofold increase in fetal distribution of topotecan compared with wild-type mice (Jonker et al. 2002). BCRP is also highly expressed lactating mammary gland. *Abcg2*$^{-/-}$ mice also showed higher plasma exposure and lower milk excretion of ciprofloxacin (Merino et al. 2006) and nitrofurantoin (Merino et al. 2005) compared with wild-type mice.

BCRP plays important role in protecting the body from a wide variety of environmental and dietary xenotoxins via limiting intestinal absorption of xenotoxins (van Herwaarden et al. 2003, 2006). van Herwaarden et al. (2006) compared plasma exposure of three dietary carcinogens 2-amino-3-methylimidazo [4,5-f]quinoline (IQ), 3-amino-1,4-dimethyl- 5H-pyrido [4,3-b]indole (Trp-P-1), and aflatoxin B1 in *Abcg2*$^{-/-}$ mice and wild-type mice. Their results showed that levels of [^{14}C]IQ, [^{14}C]Trp-P-1, and [^{3}H]aflatoxin B1 in plasma of *Abcg2*$^{-/-}$ mice were substantially higher than those in wild-type mice after both oral or intravenous administration, but their ratios of milk-to-plasma concentration were significantly lower than those of wild-type mice. 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) is also a food carcinogen and BCRP substrate. A report (van Herwaarden et al. 2003) showed that *AUC* values of PhIP following oral or intravenous administration in *Abcg2*$^{-/-}$ mice were significantly higher than wild-type mice. The recovery (26.6% of dose) of [^{14}C]PhIP in feces of *Abcg2*$^{-/-}$ mice was lower than that of wild-type mice (70.4% of dose), while recovery of [^{14}C]PhIP in urine of *Abcg2*$^{-/-}$ mice was 79.3% of dose, higher than that of wild-type mice (33.0% of dose).

BCRP substrates are often substrates of other transporter such as P-GP, indicating their common contributions to disposition of substrates. For example, de Vries et al. (2007) reported that *Mdr1a/1b*$^{-/-}$: *Abcg2*$^{-/-}$ mice showed markedly higher tissue and plasma exposure of topotecan than single *Mdr1a/1b*$^{-/-}$ or *Abcg2*$^{-/-}$ mice. *AUC* of topotecan in brains of *Mdr1a/b*$^{-/-}$ and *Abcg2*$^{-/-}$ mice was only 1.5-fold higher compared with wild-type mice, but in *Mdr1a/b*$^{-/-}$:*Abcg2*$^{-/-}$ mice, the *AUC* was increased by 12-fold. The higher *AUC* in plasma of *Mdr1a/1b*$^{-/-}$: *Abcg2*$^{-/-}$ mice was also obtained, but the extent of increase was less than in the brain. The P-GP/BCRP inhibitor elacridar fully inhibited P-GP-mediated transport of topotecan, whereas inhibition of Bcrp-mediated transport by elacridar was minimal. Similarly, Zhou et al. (2009) compared brain distribution of four substrates (flavopiridol, imatinib, PF-407288, and prazosin) in *Mdr1a/1b*$^{-/-}$: *Abcg2*$^{-/-}$, *Mdr1a/1b*$^{-/-}$, *Abcg2*$^{-/-}$, and wild-type mice using the brain-to-plasma ratios at 0.5 and 2 h following doses as index. They found that the brain-to-plasma ratios of imatinib, a better substrate of P-GP than BCRP, in *Abcg2*$^{-/-}$ mice were comparable to those in wild-type mice, whereas the brain-to-plasma ratios in *Mdr1a/1b*$^{-/-}$ and *Mdr1a/1b*$^{-/-}$: *Abcg2*$^{-/-}$ mice were more than 4- and 28-fold of those than in wild-type mice at both time points,

respectively. The Bcrp-specific substrate PF-407288 exhibited comparable brain-to-plasma ratios in *Mdr1a/1b*$^{-/-}$ and *Abcg2*$^{-/-}$ mice and slightly but significantly increased brain-to-plasma ratios in *Mdr1a/1b*$^{-/-}$: *Abcg2*$^{-/-}$ mice compared with those in wild-type mice. All these demonstrated that P-GP and Bcrp at BBB function synergistically to limit the brain penetration of shared substrates (Zhou et al. 2009).

BCRP is believed to have important physiological and pathophysiological functions in tissue and cellular protection and in mediating homeostasis of physiological substrates. Jonker et al. (2002) reported that *Abcg2*$^{-/-}$ mice displayed a new type of protoporphyria which are frequently associated with skin photosensitivity in patients due to increases in intracellular levels of porphyrins. The *Abcg2*$^{-/-}$ mice became extremely sensitive to the dietary, chlorophyll breakdown product pheophorbide A, resulting in severe, sometimes lethal phototoxic lesions on light-exposed skin. All *Abcg2*$^{-/-}$ mice (but not wild-type) progressively developed phototoxic lesions when fed with alfalfa (*Medicago sativa*) leaf (10% and 20%). Plasma levels of pheophorbide A was 24-fold higher in *Abcg2*$^{-/-}$ mice fed with 20% alfalfa food compared with a "normal" food. But, plasma levels of pheophorbide A in wild-type mice were undetectable. In compliance, *Abcg2*$^{-/-}$ mice were 100-fold more sensitive to pheophorbide A-induced phototoxicity than wild-type mice (Jonker et al. 2002).

2.4.3.2 BCRP and Side Population

One physiological role of BCRP is that BCRP is highly expressed in a "side population" phenotype, which is enriched for stem and progenitor cells in hematopoietic tissues. The side population cells character low cellular accumulation of the dye Hoechst 33342 due to high expression of BCRP. Interestingly, BCRP is highly expressed in hematopoietic progenitors and silenced in differentiated hematopoietic cells, demonstrating a role of BCRP in early hematopoiesis (Scharenberg et al. 2002). Several studies (Fatima et al. 2012; Huls et al. 2009) have been demonstrated that the side population cells also resides in other nonhematopoietical organs, such as the spleen tissue, umbilical cord blood, brain, kidney, heart, intestine, skin, and lungs. High BCRP expression is a general characteristic of various stem cell populations; thus BCRP may be served as a stem cell marker (Fatima et al. 2012). Although physiological role of BCRP in side population cells is not clearly understood, Zhou et al. (2002) reported that the disruption of the *ABCG2* gene resulted in the loss of the number of side population cells in bone marrow and skeletal muscle. The hematopoietic cells from *Abcg2*$^{-/-}$ mice were more sensitive to mitoxantrone, suggesting its protective role against cytotoxic substrates. Mouse studies (Krishnamurthy et al. 2004) demonstrated that Bcrp confers a strong survival advantage under hypoxic conditions. Under hypoxia, progenitor cells from *Abcg2*$^{-/-}$ mice had a reduced ability to form colonies compared with wild-type mice. In accordance, levels of the heme precursor and protoporphyrin IX in erythrocyte were increased tenfold in *Abcg2*$^{-/-}$ mice. Blocking BCRP function in wild-type progenitor cells also markedly reduced survival under hypoxic conditions. Blocking heme biosynthesis reversed the hypoxic

susceptibility of *Abcg2*$^{-/-}$ progenitor cells. Another report showed that transplantation with wild-type bone marrow cured the protoporphyria and reduced the phototoxin sensitivity of *Abcg2*$^{-/-}$ mice (Jonker et al. 2002). These findings suggest that cells can, upon hypoxic demand, use BCRP to reduce heme or porphyrin accumulation (Krishnamurthy et al. 2004). BCRP is present on the plasma membrane of mature red blood cells, and its expression was reported to be sharply upregulated during erythroid differentiation (Zhou et al. 2005). The erythroid cells expressing BCRP had significantly lower intracellular levels of protoporphyrin IX, suggesting the modulation of protoporphyrin IX level by BCRP. This modulating activity was abrogated by Ko143, implying that protoporphyrin IX may be a BCRP substrate and BCRP protein functions in mature red blood cells to decrease intracellular protoporphyrin IX levels.

BCRP is highly expressed in hematopoietic progenitors and silenced in differentiated hematopoietic cells, indicating a role of BCRP in both early hematopoiesis and human hematopoietic development. Data from transduced umbilical cord blood-derived early hematopoietic cells showed that BCRP increased the number of clonogenic progenitors but impaired the development of CD19^{+} lymphoid cells. In transplanted NOD/SCID mice, the Bcrp decreased the number of human B-lymphoid cells, resulting in an inversion of the lymphoid/myeloid ratio. BCRP also enhanced the proportion of CD34^{+} progenitor cells and enhanced the most primitive human progenitor pool. These results indicate that balanced expression of BCRP is crucial for normal human hematopoietic development and that its constitutive expression affects the behavior of early human progenitors and their development into more mature cell stages (Ahmed et al. 2008).

2.4.3.3 BCRP and Alzheimer's Disease

BCRP may act as a gatekeeper at the blood-brain barrier for amyloid β1–40 peptides. Xiong et al. (2009) reported that BCRP was significantly upregulated in the brains of Alzheimer's disease (AD) with cerebral amyloid angiopathy (AD/CAA) compared to age-matched controls. The increased BCRP expression was also confirmed in the brain of transgenic Alzheimer's mouse models Tg-SwDI and 3XTg. The real mechanism increasing BCRP expression by AD/CAA was unclear; in vitro study showed that conditioned media from amyloid β-activated microglia strongly induced BCRP expression. *Abcg2*$^{-/-}$ mouse studies showed that more amyloid β1-40, but not the scrambled Aβ40-1, peptides were transported into the brain (Xiong et al. 2009; Do et al. 2012; Shen et al. 2010). In vitro study also demonstrated (Do et al. 2012) that efflux of [^{3}H] amyloid β1-40 from HEK293 cell expressing BCRP was significantly greater (1.6 times) than that from parent HEK293 cells. In consistence, brain uptake clearance of [^{3}H] amyloid β1-40 in *Mdr1a/1b*$^{-/-}$ mice, but not in *Mdr1a/1b*$^{-/-}$: *Abcg2*$^{-/-}$ mice, was significantly increased by elacridar, confirming that Bcrp can mediate the efflux of [^{3}H] amyloid β1-40 from the mouse brain to the blood across the BBB. In vitro study showed that 6 h treatment with 2 μM H_2O_2 strongly stimulated the activities of α-, β-, and γ-secretases in N2a cells and that overexpression of BCRP in cells significantly inhibited the activities of all three

secretases stimulated by ROS (Shen et al. 2010). These results suggest that BCRP may act as a gatekeeper at the BBB that prevents circulatory Aβ peptides from entering into the brain. In addition, data from cell model demonstrated that BCRP enhanced the antioxidant capacity of the cells and inhibited the expression of inflammatory genes induced by ROS, further inferring a potential protective role of BCRP in Alzheimer's neuroinflammatory response (Shen et al. 2010).

2.4.3.4 BCRP and Hyperuricemia

BCRP is a high-capacity urate transporter, showing a physiological role in urate homeostasis via both renal and extrarenal urate excretion. The Q141K (*rs2231142*) variant resulting from the BCRP *c.421C > A* (substituting a glutamine for lysine) was reported to show decreased plasma membrane expression and decreased ATPase activity compared to wild-type (Morisaki et al. 2005). This variant was found with high frequency in Chinese (35%) or Japanese (35%) descent but less frequently in African American (2–5%), Hispanic (10%), European (11–14%), and Middle Eastern (13%) descent. Subjects carrying the variant are likely at an increased risk for developing gout due to reduced activity of BCRP for renal and extrarenal elimination of uric acid (Matsuo et al. 2011; Nakayama et al. 2011; Ichida et al. 2012). *Q126X* (*rs72552713*), a nonfunctional variant (Tamura et al. 2006), may also contribute to hyperuricemia. For example, Nakayama et al. (2014) investigated association of hyperuricemia and BCRP function. The participants were divided into four groups by the combination of common dysfunctional variants of BCRP, nonfunctional Q126X, and half-functional Q141K as follows: full function (Q126X: Q/Q; Q141K: Q/Q), 3/4 function (Q126X: Q/Q; Q141K: Q/K), 1/2 function (Q126X: Q/X; Q141K: Q/Q or Q126X: Q/Q; Q141K: K/K), and ≤ 1/4 function (Q126X: X/X; Q141K: Q/Q or Q126X: Q/X; Q141K: Q/K). The results showed that 53.3% of patients among tested 5005 hyperuricemia patients showed BCRP dysfunction and that the population attributable risk percent for hyperuricemia was about 30%, which was much higher than those of the other typical environmental risks. Moreover, the levels of serum uric acid significantly increased as the BCRP function decreased. Interestingly, Ichida et al. (2012) reported that contrary to the general understanding that BCRP dysfunction led to decreased renal urate excretion, BCRP dysfunction significantly increased not decreased the patient's urinary urate excretion based on data from 644 male outpatients with hyperuricemia including 575 gout cases. The mean urinary urate excretion level was increased from 24.0 mg. h^{-1}/1.73 m^2 for patients with full ABCG2 function to 34.3 mg.h^{-1}/1.73 m^2 for patients with severe dysfunction (≤1/4 function), showing an increase of 42.9%. The frequency of "overproduction" hyperuricemia (urinary urate excretion >25 mg h^{-1}/ 1.73m^2) was also increased along with decreases in BCRP function; the risk ratios (Ichida et al. 2012) of "overproduction" hyperuricemia by BCRP dysfunction were estimated to be 1.36 for 3/4 function, 1.66 for 1/2 function, and 2.35 for ≤1/4 function, respectively.

The marked association of BCRP dysfunction with an increased serum uric acid levels was also found both in end-stage renal disease (hemodialysis) and acute gastroenteritis patients. Moreover, the mean serum uric acid levels of the acute period (8.8 mg/dl) in acute gastroenteritis patients was higher than that of recovery period (4.7 mg/dl) due to impaired intestinal excretion, which is inferring pathophysiological role of BCRP in acute gastroenteritis (Matsuo et al. 2016). It was consistent with findings in patients; the *Abcg2*$^{-/-}$ mice showed significantly higher serum urate levels and urinary urate/creatinine ratio than wild-type mice, but urate excretion from the intestine was significantly decreased, which is less than a half of wild-type mice (Ichida et al. 2012; Hosomi et al. 2012). In wild-type mice, the urinary urate excretion and intestinal excretion pathways contribute approximately two-thirds, and one-third of the total urate excretion, respectively, inferring important roles of BCRP in intestinal urate excretion (Takada et al. 2014). Accordingly, increased serum uric acid levels in patients with BCRP dysfunction can be partly explained by the decreased excretion of urate from the intestine (Ichida et al. 2012).

2.4.3.5 BCRP and Cancer

Several studies have demonstrated that BCRP is associated with poor outcome in cancer and that BCRP expression may be regarded as a prognostic factor for some cancers. Both Benderra et al. (2005) and Damiani et al. (2006) reported that AML patients with high BCRP activity had a shorter disease-free survival and shorter overall survival compared with patients with low BCRP activity. Allogeneic stem cell transplantation is considered the recommended postinduction therapy for AML, but relapse still occurs in a consistent part of patients and remains the major cause of treatment failure after allogeneic stem cell transplantation. A report (Damiani et al. 2015) demonstrated that following undergoing allogeneic stem cell transplantation, the AML patients with high BCRP expression had lower free survival and increased cumulative incidence of relapse, mainly in terms of higher relapse rates compared with negative BCRP patients. Similarly, Sicchieri et al. (2015) reported that the presences of positive BCRP cells within the primary of breast cancer may predict both formation of mammospheres and patient's response to neoadjuvant chemotherapy. The positive rates of BCRP expression in 106 pancreatic ductal adenocarcinoma, 35 peritumoral tissues, 55 benign pancreatic tissues, and 13 normal pancreatic tissues were reported also to be 57.5%, 28.6%, 16.4%, and 0.0%, respectively. In parallel, the higher positive rates of BCRP expression were observed in cases with poorly differentiated tumors, lymph node metastasis, invasion to surrounding tissues and organs, and TNM stage II/IV stage disease, accompanied by shorter disease-free survival (Yuan et al. 2015). Ota et al. (2009) reported that among 156 stage 4 NSCLC patients, 51% of the patients prior to platinum-based chemotherapy exhibited higher expression of BCRP. BCRP expression was reported to be associated with shorter survival, but not with response to platinum-based chemotherapy nor progression-free survival. Yoh et al. (2004) reported that positive rate of BCRP was 46% in tumor specimens of 72 stage IIIB or IV NSCLC patients prior to

chemotherapy. The response rate (44%) to chemotherapy of patients with BCRP-negative tumors was higher than that (24%) of patients with BCRP-positive tumors. In addition, the patients with BCRP-positive tumors had a significantly shorter progression-free survival and overall survival. Interestingly, although lung cancer patients prior to therapy showed that lower expression of BCRP in chemonaïve metastatic cells of small-cell lung cancer (SCLC) than that of NSCLC, SCLC patients with high BCRP expression were associated with poor overall survival (Rijavec et al. 2011). In consistence, BCRP-positive rates in 130 SCLC patients were reported to be 37%, and BCRP expression was significantly associated with performance status, response, and progression-free survival (Kim et al. 2009a).

BCRP polymorphisms affect clinical efficacy/toxicity of chemotherapy. Imatinib is often introduced as the standard treatment for gastrointestinal stromal tumor. However, Koo et al. (2015) reported that BCRP 421C > A (Q141K) was associated with progression-free survival rate following imatinib treatment. The 5-year progression-free survival rate in patients with the AA genotype of *ABCG2 421C > A* was significantly superior to that of patients with CC/CA genotypes. A study on 125 AML Caucasian patients receiving chemotherapy showed that 26 patients displayed *ABCG2 421C > A* variant, while 99 patients were wild-type BCRP. Following treatment with idarubicin-based chemotherapy, patients with low ABCG2 and wild-type gene had a longer a 3-year overall survival compared to patients with *ABCG2 421C > A* or with high ABCG2 expression (Tiribelli et al. 2013). Similarly, sunitinib is used as the first-line treatment for advanced renal cell carcinoma, and its main adverse reaction was thrombocytopenia. Low et al. (2016) reported that *ABCG2 421C > A* functional variant is significantly associated with sunitinib-induced severe thrombocytopenia. Skin rash and diarrhea are prominent adverse events of gefitinib treatment, which occurred in more than half of patients treated with gefitinib. A report showed that 7 (44%) of 16 patients heterozygous for *ABCG2 421C > A* developed diarrhea versus only 13 (12%) of 108 patients homozygous for the wild-type sequence. However, the association between the *ABCG2 421C > A* variant and gefitinib-induced diarrhea was not found in Italy NSCLC patients (Lemos et al. 2011). In addition, *ABCG2 421C > A* variant was not associated with skin toxicity (Cusatis et al. 2006; Tamura et al. 2012). Another SNP, *ABCG2 34G > A*, was statistically associated with occurrence of skin rash (Tamura et al. 2012); 13 of the 32 patients with GA or AA genotype of *ABCG2 34 G > A* developed grade 2 or worse skin rash, whereas only 10 of 51 patients with wild-type BCRP. Lemos et al. (2011) reported that patients (50%) carrying a TT genotype of *ABCG2 15622C > T* genotype or harboring at least one TT copy in the *ABCG2* (*1143C > T*, *15622C > T*) haplotype developed significantly more grade 2/3 diarrhea. Kim et al. (2009b) investigated resistance of 229 chronic myeloid leukemia patients to imatinib therapy. Their results showed that the GG genotype of *ABCG2 34G > A* was associated with a lower complete cytogenetic response, whereas the non-AA genotype for *ABCG2 421C > A* was also associated with a lower major molecular response. The linkage disequilibrium between these two SNPs was significant, which may explain why both these SNPs associate with response to imatinib mesylate, either complete cytogenetic response or major molecular response.

ABCG2 SNPs also affect progressing cancers. Ghafouri et al. (2016) reported that A allele of *ABCG2 421C > A* polymorphism was significantly higher in breast cancer patients than in healthy subjects and that patients with AA genotype of *ABCG2 421C > A* were at higher risk of progressing breast cancer, although patients with A allele of BCRP had complete response to chemotherapeutic agents. In consistence, Wu et al. (2015) reported that GA/AA genotype of *ABCG2 34G > A*, AA genotype of *ABCG2 421C > A*, and haplotypes 34A-421C and 34G-421A were significantly associated with increased risk for developing breast carcinoma. Moreover, AA genotype of *BCRP 421C > A* had a significant enhanced therapeutic response in patients with neoadjuvant anthracycline-based chemotherapy. The patients carrying AA genotype of *ABCG2 34G > A* displayed a longer overall survival in estrogen receptor-positive patients or progesterone receptor-positive patients after postoperative anthracycline-based chemotherapy. These results suggest that the *ABCG2* SNP may be a candidate pharmacogenomic factor to assess susceptibility and prognosis for breast carcinoma patients (Wu et al. 2015; Ghafouri et al. 2016). Similarly, a study (Hu et al. 2007) on 156 diffuse large B-cell lymphoma patients and 376 control subjects revealed an increased risk of diffuse large B-cell lymphoma associated with variant *ABCG2 421C > A* genotypes (CA and AA) compared with the wild-type CC genotype. The increased risk was more evident in younger patients ($\leq$50 years). CC genotypes of *ABCG2 421C > A* was significantly associated with poorer survival in younger patients ($\leq$50 years) or with bulky tumor. Furthermore, patients carrying AA genotype of *ABCG2 34G > A* also displayed worse survival compared with those carrying GG/GA genotypes. The combined effects of A*BCG2 34G > A* and 421C > A on the overall survival were found. Compared with patients carrying *ABCG2* 34 (GG + GA)-421 (AA+CA) genotype, the individual with 34AA-421CC displayed the worst survival.

It was noted that some studies gave contrast reports. For example, Salimizand et al. (2016) reported that CC genotype of *ABCG2 421C > A* was significantly higher in chronic myeloid leukemia patients (about 63%) compared to controls (25%). Attractively, frequency (70%) of carriers of A allele of *ABCG2 421C > A* in healthy subjects was significantly higher than that (33.6%) of patients. And, AA genotype of *ABCG2 421C > A* was associated with a lower risk of chronic myeloid leukemia development. Similarly, Korenaga et al. (2005)'s report showed that the frequency of the CC genotype of *ABCG2 421C > A* was significantly higher in renal cell carcinoma patients than that in control subjects, indicating that carriers with the CC genotype of *ABCG2 421C > A* are at risk of developing nonpapillary renal cell carcinoma and that BCRP is a candidate renal cell carcinoma susceptibility gene (Korenaga et al. 2005). Although the molecular mechanism for the relationship between BCRP and carcinogenesis remains to be resolved, the concentrations of pheophorbide A, folate, or estradiol in the body may be maintained at relatively low levels in individuals with the CC genotype of *ABCG2 421C > A*, resulting in a higher risk of cancer development, which needs further investigation.

Several studies have demonstrated that some cancers themselves affect BCRP expression. A report (Gupta et al. 2006) showed that levels of BCRP mRNA were decreased by sixfold in colon cancer compared with normal colorectal tissue. The

downregulation of BCRP mRNA and protein was also evident in cervical cancer. Similar decreases in BCRP mRNA were also found in other cancer tissues such as the breast, ovary, liver, lung, and small intestine. In consistence, Dietrich et al. (2011) reported that levels of BCRP protein in human colorectal adenomas was significantly decreased to 28% of adjacent healthy tissue. These results were in line with data from adenomas of Apc^{Min} mice, a mouse model for intestinal and mammary tumorigenesis. The protein expression of Bcrp in adenomas was only 58% of surrounding healthy tissue. A higher carcinogen concentration in adenomas of Apc^{Min} mice (181% of normal tissue) including immunohistochemical detection of PhIP-DNA adducts was found following oral administration of PhIP, suggesting that downregulation of Bcrp expression led to higher carcinogen concentrations in colorectal adenomas of mice and men, promoting the adenoma-carcinoma sequence by higher genotoxic effects (Dietrich et al. 2011).

BCRP protein is also expressed inside the nucleus of human cancer cells, where it binds to the E-box of E-cadherin promoter and regulates transcription of this gene. Increased expression of BCRP causes an increase of E-cadherin and attenuates cell migration, whereas knockdown of BCRP downregulates E-cadherin and enhances cell motility. In mice, xenografted A549 cells that have less Bcrp are more likely to metastasize from the subcutaneous inoculation site to the internal organs. However, for the cancer cells that have already entered the blood circulation, an increased level of BCRP, and correspondingly increased E-cadherin, may facilitate circulating cancer cells to colonize at a distant site and form a metastatic tumor, inferring novel role for nuclear BCRP that functions as a transcription regulator and participates in modulation of cancer metastasis (Liang et al. 2015). BCRP is also distributed in mitochondria, where BCRP regulates the content of 5-aminolevulinic acid-mediated protoporphyrin IX in mitochondria. Ko143, a specific inhibitor of BCRP, was reported to enhance mitochondrial protoporphyrin IX accumulation (Kobuchi et al. 2012). Mitoxantrone accumulation was significantly reduced in mitochondria and in cells that overexpress BCRP, in comparison to parental drug-sensitive cells. The specific inhibitor of BCRP, FTC, significantly increased the accumulation of mitoxantrone in comparison with basal conditions in both whole cells and in mitochondria of BCRP-overexpressing cell lines (Solazzo et al. 2009).

2.5 ABC Genes and Human Genetic Disease

2.5.1 Cystic Fibrosis and CFTR

Cystic fibrosis (Chiaw et al. 2011**;** Bonadia et al. 2014; Fanen et al. 2014) is a common autosomal recessive disorder within the Caucasian population with high lethality and morbidity, reaching frequencies ranging from 1:2500 to 1:4500. The disease is much less common in African and Asian populations. Cystic fibrosis is caused by mutations in CFTR/*ABCC7*, primarily expressed in apical membranes of epithelial tissues such as the airways, intestine, pancreas, and sweat ducts, where it

controls transepithelial salt and fluid movement. Almost 2000 mutations, including deletions, missense, frameshift, and nonsense mutations, have been identified in the *ABCC7* gene, which have been linked with the cystic fibrosis phenotype. Most mutations are extremely rare. The exception is the deletion of the three base pairs, which result in the loss of the phenylalanine residue at position 508 of the CFTR protein sequence (termed F*508del*).

Cystic fibrosis patients frequently suffer from exocrine pancreatic insufficiency, intestinal obstruction, male sterility, and other effects. Mortality in cystic fibrosis is primarily caused by chronic lung infection. It is through CFTR that some bacterial toxins such as cholera and *Escherichia coli* cause increase fluid flow in the intestine, resulting in diarrhea. CFTR is also the receptor for *Salmonella typhimurium* and implicated in the innate immunity to *Pseudomonas aeruginosa.* Importantly, it is the lung infections with opportunistic microbial pathogens, such as the bacteria *Pseudomonas aeruginosa* and *Burkholderia cepacia* and the fungi *Aspergillus fumigatus*, and consequently respiratory degeneration and failure, ultimately leading to death.

2.5.2 Adrenoleukodystrophy

X-linked adrenoleukodystrophy (X-ALD) is the most common peroxisomal neurodegenerative disease with a frequency of 1 in 21,000 males in the USA, due to the impaired peroxisomal β-oxidation of very long-chain fatty acids, leading to accumulation of very long-chain fatty acids (Dean et al. 2001; Kemp et al. 2012; Engelen et al. 2014; Berger et al. 2014). All patients have mutations in the *ABCD1* gene. *ABCD1* gene encodes adrenoleukodystrophy protein (ALDP), which is located in the peroxisomal membrane protein, and transports its substrates (CoA-activated very long-chain fatty acids, such as C26:0-CoA, C24:0-CoA, or C22:0-CoA) from the cytosol into the peroxisome under ATP consumption where these fatty acids are degraded by peroxisomal β-oxidation. ALDP/ABCD1 deficiency impairs the peroxisomal beta-oxidation of very long-chain fatty acids and facilitates their fatty chain elongation, resulting in accumulation of very long-chain fatty acids in plasma and tissues. The accumulated very long-chain fatty acids lead to death of neural cells via a combination of a disturbance in calcium homeostasis and mitochondrial dysfunction.

2.5.3 Sulfonylurea Receptor

The *ABCC8* (SUR1), an ABC transporter, is a high-affinity receptor for the drug sulfonylurea (Flanagan et al. 2009; Aittoniemi et al. 2009). SUR1 is unique among ABC proteins in that it serves as a channel regulator, forming a tightly associated octameric K_{ATP} channel complex in which four Kir6.2 subunits form a central pore surrounded by four SUR1 subunits. SUR1 affects Kir6.2 via enhancing the open

probability, increasing the channel ATP sensitivity, conferring sensitivity to activation by Mg-nucleotides such as MgATP and MgADP, or endowing the channel with sensitivity to therapeutic drugs. These therapeutic drugs bind directly to SUR1 to modulate K_{ATP} channel activity: for example, sulphonylureas inhibit but diazoxide (K-channel openers) activate the channel. Mutations in SUR1 can cause insulin secretion disorders such as neonatal diabetes and hyperinsulinism. Loss-of-function SUR1 mutations is considered to be associated with hyperinsulinemic hypoglycemia of infancy, characterized by excess insulin release for the degree of hypoglycemia. Interestingly, Baier et al. (2015) reported loss-of-function *ABCC8 R1420H* variant with a high carrier rate (3.3%) in a southwestern American Indian community. *R1420H* carriers have increased birth weight presumably due to fetal hyperinsulinemia and a twofold increased risk of diabetes with a younger age of onset. On the contrast, gain-of-function SUR1 mutations are linked to neonatal diabetes due to decreases in insulin secretion. Some of these mutations also lead to more severe syndromes such as developmental delay and muscle weakness or to developmental delay, epilepsy, muscle weakness, and dysmorphic features.

Reports on association between mutations in SUR1 and type 2 diabetes are often contradictory (Haghverdizadeh et al. 2014). For example, in *ABCC8 rs757110*, of seven reports, only two reports in Japan and Britain showed association between this variant and risk of type 2 diabetes. For *ABCC8 rs1799854*, of 17 studies in Asian and Caucasian populations, only 6 reported association between this SNP and risk of type 2 diabetes and gestational diabetes in Japanese, Chinese, French, Turkish, and Dutch populations. For *ABCC8 rs1799859*, of nine studies, two reports in the USA and Japan showed no association between this SNP and type 2 diabetes, and for *ABCC8 rs1801261*, of six studies, only two reports showed association between this variant and risk of type 2 diabetes in Danish and Canadian populations. These results infer that association between mutations in SUR1 and type 2 diabetes should be confirmed.

2.5.4 Bile Salt Transport Disorders

Bile is a largely (~95%) aqueous fluid that is produced by the hepatocyte in the liver and released into the biliary system of ducts. Bile is a complex mixture of endogenous solid constituents, including bile salts, bilirubin, phospholipids, cholesterol, amino acids, steroids, enzymes, porphyrins, vitamins, xenobiotics, and environmental toxins. Secretion of bile salts, phosphatidylcholine, and cholesterol is mediated by ABCB11 (BSEP), ABCB4 (MDR3), and ABCG5/ABCG8, respectively. Excretion of organic anions is mediated by other members of the ABC transporter such as MRP2. In addition, ATP8B1, a P4 P-type ATPase, is essential for a proper composition of the canalicular membrane and thus for normal bile flow. Their defects are responsible for several forms of progressive familial intrahepatic cholestases (PFICs), which are identified into three subtypes PFIC1, PFIC2, and PFIC3 based on genetic and molecular abnormalities.

PFIC1 PFIC1 is considered to be caused by ATP8B1 deficiency (Stapelbroek et al. 2010; Nicolaou et al. 2012). ATP8B1 is a P-type ATPase and specifically translocates phosphatidylserine from the outer to the inner leaflet of plasma membranes, leading to the outer leaflet to be enriched in phosphatidylserine, sphingomyelin, and cholesterol. Cholesterol has a high affinity for sphingomyelin, and both are thought to be preferentially located in laterally separated microdomains. These microdomains are considered to be essential for the maintenance of membrane integrity in the presence of high concentrations of detergent bile acids and normal function of transmembrane transporters. Disruption of lipid asymmetry and reduction of cholesterol content in the apical membrane impairs protection against the detergent action of bile salts and decreases the function of resident proteins such as BSEP. ATP8B1 deficiency is linked to several cholestatic liver diseases including PFIC1, benign recurrent intrahepatic cholestasis type 1, and intrahepatic cholestasis of pregnancy. PFIC1 is by far the most severe. Its common outcome is cirrhosis and end-stage liver disease within the second decade. ATP8B1 is expressed in other tissues such as apical membranes of pancreatic acinar cells, enterocytes, and cochlear hair cells of the inner ear. Therefore, PFIC1 patients are often accompanied by extrahepatic symptoms including intractable diarrhea, sensorineural hearing loss, and pancreatitis.

PFIC2 PFIC2 is caused by mutation to BSEP (Oude Elferink and Paulusma 2007; Benzimra et al. 2013; Nicolaou et al. 2012; Stapelbroek et al. 2010; Soroka and Boyer 2014; Kubitz et al. 2012). BSEP is highly expressed on the liver canalicular membrane and has been shown to be the major bile salt export pump. Their mutations result in a range of mild to severe, progressive forms of intrahepatic cholestasis (known as the BSEP deficiency syndrome). Mutations in BSEP are also associated with benign recurrent intrahepatic cholestasis type 2, drug-induced cholestasis, and intrahepatic cholestasis of pregnancy. Patients with BSEP mutations are also at risk for hepatocellular carcinoma. Mouse experiment demonstrated that Bsep knockout led only to mild cholestasis with substantial bile flow and upregulated P-GP genes and knockout of the three genes (Bsep, mdr1a/1b) led to a significantly more severe phenotype with impaired bile formation, jaundice, flaccid gallbladder, and increased mortality. The triple-knockout mouse was the most severe genetic model of intrahepatic cholestasis. These findings demonstrated critical compensatory mechanism of P-GP, which reduces the severity of cholestasis in Bsep knockout mice (Wang et al. 2009).

Drugs can cause drug-induced cholestasis via different mechanisms on BSEP. Some drugs such as cyclosporine A, glibenclamide, troglitazone (and its sulfated metabolite), bosentan (and its metabolites), and nefazodone are potent inhibitors of BSEP. Other drugs such as estrogen 17β-estradiol led to drug-induced cholestasis via suppressing BSEP expression (Chen et al. 2015). Drug-induced liver injury has been the reason for withdrawal of several drugs from the market, indicating that it is important to investigate BSEP inhibition by candidate drugs that has high importance in drug discovery and development.

PFIC3 PFIC3 is caused by ABCB4 mutation (Nicolaou et al. 2012; Stapelbroek et al. 2010; Benzimra et al. 2013; Oude Elferink and Paulusma 2007). ABCB4, a 1279-amino acid transmembrane protein, is located in canalicular membrane and translocates phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane of the hepatocyte, which mediates excretion of conjugated bile salts (such as taurochenodeoxycholate, taurocholate, tauroursodeoxycholate, and glycocholate) and phosphatidylcholine into bile. The flopped phosphatidylcholine is extracted by the bile salts in the canaliculus to form a mixed micelle that reduces bile salt detergent activity. Mutations in the ABCB4 gene develop PFIC3 due to decrease in biliary phosphatidylcholine. PFIC3 patients have reduced concentration of phosphatidylcholine in bile. On the contrast to patients with PFIC1 and PFIC2, serum γ-glutamyltranspeptidase levels are elevated. Liver histology reveals fibrosis and marked bile duct proliferation. Mutations in the ABCB4 gene are also associated with intrahepatic cholestasis of pregnancy, drug-induced cholestasis, transient neonatal cholestasis, or isolated and recurrent intrahepatic cholesterol gallstones. Some drugs such as itraconazole induced cholestatic liver injury via inhibiting ABCB4 function (Yoshikado et al. 2011). In addition to BSEP inhibition, the possibility of ABCB4 inhibition should be taken into account for a better understanding of the mechanism of drug-induced cholestasis.

2.5.5 Retinal Degeneration and ABCA4

ABCA4 protein is a ~250 kDa ABC transporter expressed in rod and cone photoreceptors of the vertebrate retina (Dean et al. 2001; Molday et al. 2009; Tsybovsky et al. 2010; Quazi and Molday 2014). ABCA4 efflux rhodopsin, retinal, and their conjugates with phospholipids. ABCA4 also mediates transport of *N*-retinylidene-phosphatidylethanolamine, a Schiff base product of the reaction of all-*trans*-retinal with phosphatidylethanolamine, to the cytoplasmic side of the disk membrane, where it can dissociate, allowing all-*trans*-retinal to reenter the visual cycle. ABCA4 also would reduce formation of potentially harmful diretinal compound di-retinoid-pyridinium-phosphatidylethanolamine via decreasing accumulation of *N*-retinylidene-phosphatidylethanolamine and all-*trans*-retinal inside the disk. Thus, mutations in the *ABCA4* gene progressively accumulate large quantities of di-retinoid-pyridinium-phosphatidylethanolamine in the retinal pigment epithelium, in turn impairing retinal pigment epithelium cells, ultimately leading to multiple eye disorders including Stargardt disease and age-related macular degeneration.

2.5.6 Mitochondrial Iron Homeostasis

To date, four ABC transporters have been identified in mitochondrial, i.e., ABCB6, ABCB7, ABCB8, and ABCB10 (Zutz et al. 2009). ABCB7, ABCB8,

and ABCB10 are localized in the inner mitochondrial membrane, while ABCB6 is expressed in the outer mitochondrial membrane. The main function of ABCB7 is to keep cellular iron homeostasis via regulating iron incorporation into Fe/S clusters and heme. The mutations in *ABCB7* gene result in X-linked sideroblastic anemia with cerebellar ataxia, a disease of early life characterized by the onset of a nonprogressive cerebellar ataxia and microcytic anemia, and abnormally high levels of mitochondrial iron (Burke and Ardehali 2007). ABCB6 is a porphyrin importer. The main function of ABCB6 is to regulate de novo porphyrin synthesis. In addition, ABCB6 protects cells from certain stresses via regulating formation of hemoproteins (Lynch et al. 2009). Roles of other two ABCB8 and ABCB10 in heme metabolism need further investigation.

2.5.7 Sterol Transport Deficiencies

Tangier disease is caused by mutations in *ABCA1* gene and characterized by severe plasma deficiency or absence of high-density lipoprotein, apolipoprotein A-I, and accumulation of cholesteryl esters in many tissues such as the tonsils, peripheral nerves, spleen, liver, cornea, bone marrow, lymph nodes, thymus, and skin (Puntoni et al. 2012). ABCA1 expressed in most tissues plays a central role in cellular cholesterol homeostasis and high-density lipoprotein formation via effluxing cholesterol and phospholipids from cells to lipid-poor apolipoprotein A1 (apoA-1) to generate nascent high-density lipoprotein, a pathway termed reverse cholesterol transport. Mutations in *ABCG5* and *ABCG8* gene were linked to sitosterolemia (Yu et al. 2014). Sitosterolemia, a rare autosomal recessive disorder, is characterized by markedly elevated plasma levels of plant sterols and modest increases in plasma cholesterol due to the hyperabsorption of these sterols from the small intestine and a reduced excretion into the bile. ABCG5 and ABCG8 are half transporters. They must form the heterodimer to obtain sterol transport functionality. The two transporters are expressed almost exclusively on the brush border membrane of enterocytes and the canalicular membrane of hepatocytes. In the intestine, ABCG5 and ABCG8 transport cholesterol and plant sterols from enterocytes into the gut lumen for fecal disposal. They are considered to be predominant determinants of intestinal cholesterol and plant sterols absorption. In the liver, ABCG5 and ABCG8 can promote cholesterol and plant sterol elimination from the body through hepatobiliary secretion. Thus mutations in either of the two genes cause sitosterolemia, a condition in which cholesterol and plant sterols accumulate in the circulation leading to premature cardiovascular disease.

Other ABC transporters such as ABCG1 and ABCG4 are able to promote the efflux of cholesterol to high-density lipoprotein particles, suggesting that these two genes may also be involved in cholesterol transport (Woodward et al. 2011).

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