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Guoyu Pan *Editors*

Drug Transporters in Drug Disposition, Effects and Toxicity

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Drug Transporters in Drug Disposition, Effects and Toxicity

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Preface

Drug transporters are well-recognized determinants of drug disposition and effects/toxicity. These are divided into solute carrier (SLC) family and ATP-binding cassette (ABC) family. Functionally, they mediate drug uptake into cells and export drugs out of cells, respectively.

Each transporter has a specific pattern of tissue expression. Transporters expressed in the small intestine, liver, and kidney are very important for drug disposition and drug-drug interactions, while transporters expressed in blood-tissue barriers, such as blood-brain barrier and maternal-fetal barrier, protect sensitive tissues from toxic compounds. Drug transporters are essential in maintaining cell homeostasis, and gene mutations often cause or contribute to several human genetic disorders including cystic fibrosis, neurological disease, retinal degeneration, cholesterol and bile transport defects, anemia, and drug response. Some diseases alter transporter function and expression, in turn, aggravating disease process.

Overexpressions of some ABC transporters are also potential contributors to multidrug resistance (MDR) development for anticancer drugs. Several ways are being tried to overcome MDR. One way is to develop ABC transporter inhibitor to sensitize cancer cells to chemotherapeutic drugs. Another way is to develop no substrates of ABC transporters to gain therapeutic benefit in multidrug-resistant tumors. microRNA and other epigenetic methods are also considered to be useful tools to modulate transporter expressions and functions.

This book will focus on the roles of drug transporters in drug disposition and effects/toxicity. It will also cover drug-drug interaction and recent great progress on transporters. The provided information is great for graduate students and professionals who are looking to refresh or expand their knowledge in this field.

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Chapter 1
Overview: Role of Drug Transporters in Drug Disposition and Its Clinical Significance

Xiaodong Liu

Abstract Absorption, distribution, and excretion of drugs are involved in drug transport across plasma membrane, most of which are mediated by drug transporters. These drug transporters are generally divided into solute carrier (SLC) family and ATP-binding cassette (ABC) family. These transporters not only mediate transport of therapeutic drugs across membrane but also transport various kinds of endogenous compounds. Thus besides being participated in disposal of drug and its clinical efficacy/toxicity, these transporters also play vital roles in maintaining cell homeostasis via regulating transport of endogenous compounds. This chapter will outline classification of drug transporters, their roles in drug disposal/drug response, and remote communication between tissues/organs.

Keywords Remote communication · Drug transporter-metabolism interplay · ATP-binding cassette family transporters · Solute carriers

1.1 General Consideration

Absorption, distribution, and excretion of drugs are involved in drug transport across plasma membrane, most of which are mediated by drug transporters. These drug transporters are considered to be key determinants of drug accumulation within cells, whose activities are often directly related with therapeutic efficacy, drug toxicity, and drug-drug interactions. These drug transporters are generally divided into solute carrier (SLC) family and ATP-binding cassette (ABC) family.

In humans, more than 300 SLC transporters have been identified. SLC family transporter-related drug transport mainly include organic anion-transporting polypeptides (OATPs/SLCOs), organic anion transporters (OATs/SLC22As), organic cation transporter (OCTs/SLC22As), organic cation and carnitine transporters

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(OCTNs/SLC22As), peptide transporters (PEPTs/SLC15As), and multidrug and toxin extrusions (MATEs/SLC47As). Most of SLC transporters belong to influx transporters. They mediate movement of solutes from extracellular milieu into cells, either by passive diffusion along its concentration gradient, by cotransport, or counter-transport against its concentration gradient by co-opting the concentration gradient of another solute. The gradient is ultimately derived from ATP hydrolysis-driven transporters using ATP as energy. Thus, SLC transporters are considered as facilitated transporters or secondary active transporters. Although MATEs belong to SLC family, they act as efflux transporters. These SLC transporters are widely distributed in various tissues, such as the intestine, liver, kidney, and brain (Fig. 1.1), showing key roles in drug uptake into these tissues, in turn, affecting their therapeutic effects/toxicity (Shitara et al. 2013; Roth et al. 2012).

ABC transporters belong to efflux transporters. They export drugs out of cells using ATP as driving energy. To date, 48 ABC transporter subtypes have been identified in humans, and they are further divided into seven subfamilies (ABCA to ABCG) based on their gene structure, amino acid sequence, domain organization, and phylogenetic analysis (Dean et al. 2001). At least 11 ABC transporters have been identified related to multidrug resistance (MDR) development, including P-glycoprotein (P-GP/ABCB1), multidrug resistance-associated proteins (MRPs/ABCCs), and breast cancer resistance protein (BCRP/ABCG2) (Schinkel and Jonker 2012; Slot et al. 2011). Each ABC transporter has a specific pattern of tissue expression. ABC transporters expressed in the small intestine, liver, and kidney show important roles in drug disposition and drug-drug interactions (Fig. 1.1). Some blood-tissue barriers such as blood-brain barrier (BBB), fetal-maternal barrier, and blood-eye barriers exhibit high expression of corresponding ABC transporters, protecting sensitive tissues from toxic compounds and maintaining cell homeostasis.

In general, SLC transporters and ABC transporters often share tissue distributions and overlap substrate specificities, coordinating with each other for the transport of a wide array of substrates. Moreover, interplay of the uptake transporters and efflux transporters together with phase I or II metabolism may be required for the sequential traverse of the basolateral and apical membranes. They work in concert to enable the elimination of a particular drug in the indicated tissues (Fig. 1.2a), termed as "drug transporter-metabolism interplay." For example, diclofenac is mainly extracted from the portal blood into hepatocytes via OAT2 at basolateral surface of hepatocytes, where it is metabolized to 4'-hydroxydiclofenac and 5'-hydroxydiclofenac by CYP450s or diclofenac acyl glucuronide by UDP-glucuronosyltransferases. Then these metabolites and parent drug are effluxed out of hepatocytes to bile via MRP2 and BCRP at canalicular membrane or returned to blood via MRP3 at basolateral surface of hepatocytes (Scialis et al. 2015; Lagas et al. 2010; Lagas et al. 2009; Zhang et al. 2016). Therefore, drug transporters can be regarded as completing the phase I or II enzyme-based detoxification system; drug uptake delivers the drug to the detoxification system to facilitate metabolism, whereas drug efflux decreases the load on detoxification enzymes (Kalliokoski and Niemi 2009). Similarly, some efflux transporters (such as P-GP and MATEs) are mainly expressed at luminal membranes of the renal tubules, and uptake transporters (such as OCT2 and OATs)

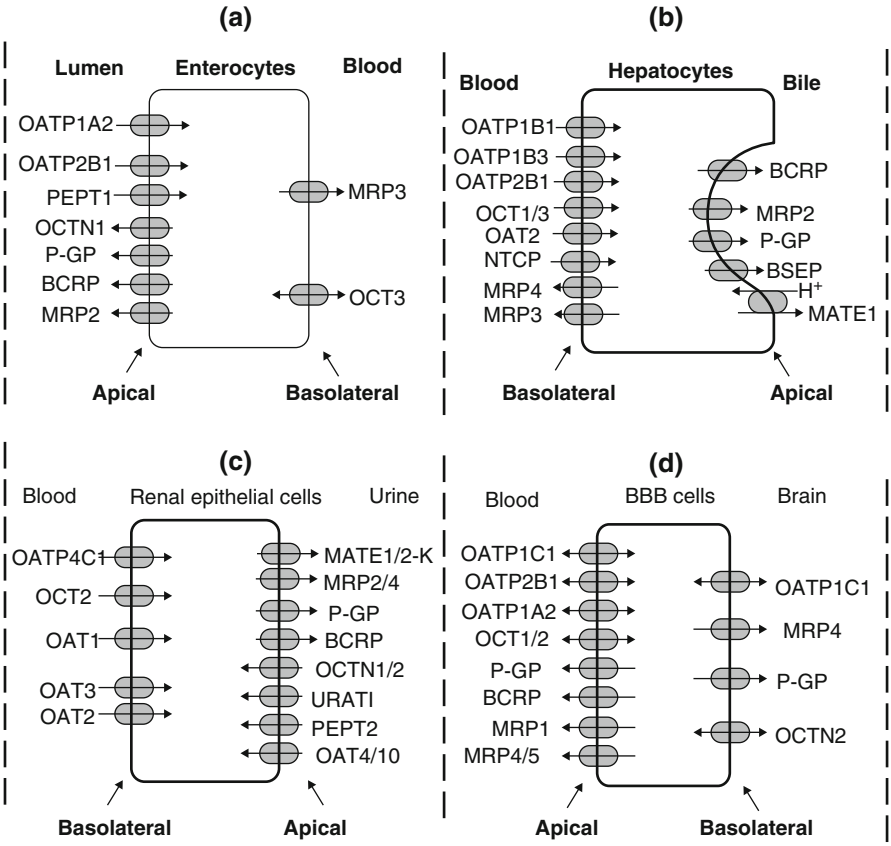

Fig. 1.1 Possible location of main transporters in the human intestine (**a**), liver (**b**), kidney (**d**), and blood-brain barrier (BBB). Symbol: *BCRP* breast cancer resistance protein, *BSEP* bile salt export pump, *MATEs* multidrug and toxin extrusions, *MRPs* multidrug resistance-associated proteins, *NTCP* sodium taurocholate co-transporting polypeptide, *OATs* organic anion transporters, *OATPs* organic anion-transporting polypeptides, *OCTNs* organic cation and carnitine transporters, *OCTs* organic cation transporters, *PEPTs* peptide transporters, and *P-GP* P-glycoprotein

are often expressed at the basolateral membrane of renal tubules; they work in series to structure an eliminatory pathway of a particular drug via urine (Fig 1.2b).

It should be noteworthy that transport of a drug is often involved in multiple SLC and ABC transporters, therefore, to define the most physiologically relevant transport pathway or pathways for a particular drug is challenging task. For instance, methotrexate can be transported by SLC transporters (such as OAT1, OAT3, OAT4, OATP1A2, and OAT1B3) and ABC transporters (such as MRP2, MRP3, MRP4, MRP5, and BCRP) (Nigam 2015). These transporters show different tissue expression patterns. In addition, they show marked differences in the affinities of the substrate. Another example is metformin. Metformin is a substrate of OCT1, OCT2, OCT3, and

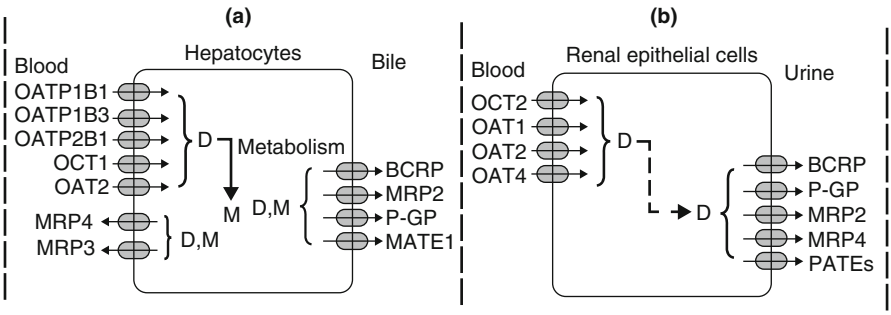

Fig. 1.2 (**a**) Role of hepatic transporters affecting hepatic uptake and excretion of a drug and the interplay of hepatic transporters with Phase I or Phase II metabolism; (**b**) Renal efflux transporters at apical surface work in concert with renal uptake transporters at basolateral surface to structure an eliminatory pathway of a particular drug via urine. BCRP, breast cancer resistance protein; P-GP, P-glycoprotein; MRPs, multidrug resistance-associated proteins; OATP1B1, organic anion-transporting polypeptide 1B1; OCTs, organic cation transporters; OATs, organic anion transporters; MATEs, multidrug and toxin extrusions; D, drug; and M, its metabolite

MATEs (Nigam 2015), which in common contribute to disposition of metformin following oral dose. Similarly, platinum antitumor agent oxaliplatin is also substrate of OCT2 and MATEs (Staud et al. 2013). OCT2 on the basolateral surface and MATEs on the apical surface of kidney proximal tubule cells work in series, mainly contributing to the urine excretion of metformin and oxaliplatin.

1.2 Physiological Roles of Drug Transporters

In addition to transport of drugs, growing data have demonstrated that these drug transporters also transport many endogenous compounds such as metabolites, nutrients, antioxidants, microbiome products, bile salts, neurotransmitters, neuroactive molecules, hormones, and signaling molecules, inferring their roles in maintenance of cell homeostasis. Their gene mutations have often contributed to several human genetic disorders including cystic fibrosis, neurological disease, retinal degeneration, cholesterol and bile transport defects, anemia, and drug response (Dean et al. 2001; Stefková et al. 2004; Quazi and Molday 2011; van der Deen et al. 2005). For example, hepatic MRP2 is involved in transport of bilirubin glucuronides, and its mutations result in Dubin-Johnson syndrome, which presents with conjugated hyperbilirubinemia (Toh et al. 1999). Mutations in *ABCB4* and *ABCG5/ABCG8* gene are responsible for progressive intrafamilial hepatic disease and sitosterolemia, respectively (Quazi and Molday 2011). Mutations in *ABCA1*gene cause Tangier disease due to defective efflux of cholesterol and phosphatidylcholine from the plasma membrane to the lipid acceptor protein apoA1. Mutations in *SLC22A5* gene (known as OCTN2) cause systemic carnitine deficiency, leading to cardiomyopathy (Tamai 2013).

SLC and ABC drug transporters also show important roles in response to pathological states. Liver injury was reported to alter expression of renal SLC and ABC drug transporters, whereas loss of kidney tissue or function could affect the expression of liver transporters (Lu and Klaassen 2008; Naud et al. 2012). Similarly, liver injury downregulated expression of P-GP and BCRP at BBB (Fan and Liu 2018; Jin et al. 2013) and intestine (Wang et al. 2017). Moreover, diabetes, epilepsy, and stroke may alter function and expression of these transporters, in turn, aggravating disease process or inducing new complication. Upregulation of P-GP expression and function by epilepsy or antiepileptic drugs become a reason leading to drug-resistant epilepsy (Jing et al. 2010). Diabetes was reported to downregulate expression of P-GP and BCRP at BBB and alter the permeability of their substrates including drugs and endogenous substances across BBB, in turn, affecting activity/toxicity of their substrates on central nervous system, which may contribute to brain complications under diabetic conditions (Liu and Liu 2014). Diabetes also induced hepatic Oatp1b2, leading to increases in hepatotoxicity of atorvastatin (Shu et al. 2016).

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). It is widely accepted that overexpressions of some ABC transporters in tumor tissues are also potential contributors to multidrug resistance (MDR) development for anticancer drugs (Dean et al. 2001).

1.3 Roles of Drug Transporters in Inter-organ Communication

SLC and ABC transporters, expressed in barrier epithelia at the interface of fluid compartments, coordinately mediate the transepithelial movement of a wide variety of substrates including nutrients, toxins, signaling molecules, neurotransmitters, and xenobiotic compounds. Many substrates are well-known signaling molecules such as cyclic nucleotides, prostaglandins, and conjugated steroids. Characteristics of both the distribution of these transporters in diverse organs and their overlapping substrate specificities suggest that these transporters may participate in a broader communication network between organs/tissues via transporting these signaling molecules. A "remote sensing and signaling" hypothesis may explain how transporters work together in remote communication between tissues and organisms and control fluctuations of these signal molecules (Wu et al. 2011; Nigam 2015). The signaling molecules generated in one organ can be secreted into the circulation via their transporters, where these signal molecules can be absorbed into targeted organs via a set of transporters and show their physiological functions. These molecules are also transported into renal epithelia cells and eliminated from the body via urine. That is to say that the renal transporters mainly maintain the organism's (whole-body blood circulation) substrate homeostasis, whereas the transporters in the targeted organs (remote) maintain the substrate homeostasis locally.

A typical example is uric acid, a primary nitrogenous end product of protein and purine metabolism in many animals including humans. Under normal physiological condition, blood levels of uric acid range between 36 mg/L and 83 mg/L. Uric acid is also a key regulator of redox states within cells and tissues which functions as an antioxidant. In addition, uric acid may also show neuroprotective activity. The elevated levels of blood uric acid were reported to reduce the risk of developing Parkinson's disease and to correlate with slower disease progression (Elbaz and Moisan 2008). However, high uric acid level is also correlated with gout, hypertension, cardiovascular disease, and renal disease (Heinig and Johnson 2006; Melinda et a1.2008). In human, the primary elimination routes of uric acid are renal and intestinal excretions, accounting for approximately 60%~65%(Hyndman et al. 2016) and 30%(Takada et al. 2014) of total urate excretion, respectively. Urate can be handled by multiple transporters such as urate transporter 1(URAT1), glucose transporter 9(GLUT9), sodium-dependent phosphate transport protein 1(NPT1), NPT4, OAT1, OAT3, OAT4, OAT10, BCRP, and MRP4 (Hyndman et al. 2016). Intestinal secretion of uric acid is mainly mediated by BCRP (Takada et al. 2014). In the kidney, URAT1, OAT4, and OAT10 are predominantly expressed on the apical membrane of the proximal tubule cells in the kidney, accounting for uric acid reabsorption from the ultrafiltrate, among which, URAT1 has been identified as one of the most important transporters for urate reabsorption from the apical side of the proximal tubule. Certain URAT1 mutations are associated with increased uric acid levels, gout, and kidney stone (Nigam et al. 2015; Reginato et al. 2012). GLUT9, OAT1, and OAT3 are expressed at basolateral membrane of proximal tubule. Main function of GLUT9 is responsible for the efflux of reabsorbed urate from the proximal tubule cells back to the blood, although in vitro data demonstrated that GLUT9 is also capable of importing urate. OAT1 and OAT3 have been considered the primary candidates for uptake of urate, but no genetic data support their roles. BCRP, NPT1, NPT4, and MRP4 have all been localized to the apical side of the proximal tubule and serve as apical secretory transporters for urate.

In addition to the kidney, each of these transporters may also have other preferred organs of expression. For example, GLUT9 is also expressed in the ovary and bone; NPT1 and MRP4 are in the liver; NPT4 is in the brain; and OAT4 is in the placenta, and BCRP is in intestine. These distinctive expression patterns suggest that urate levels in some particular organs and in the kidney are coordinately regulated via a remote communication of urate concentration which is potentially tied to expressions and functions of these transporters (Nigam et al. 2015) (Fig. 1.3).

Handling of prostaglandins (PGs) is another example how a signaling pathway and drug transporter may be intertwined. PGs are produced in most organs and tissues. They are considered to be local hormones acting in a paracrine or autocrine fashion. They are also released to circulation, showing remote communication. It is now clear that transporters, particularly MRP4, mediate the release of many PGs (Klaassen and Aleksune 2010). Other ABC transporters such as ABCA1 and MRP2 (de Waart et al. 2006; Liu et al. 2011) may also play a role in the release of cellular PGs. On the other hand, multiple SLC transporters are involved in the cellular uptake of certain PGs (Klaassen and Aleksune 2010). The first PG transporter identified is

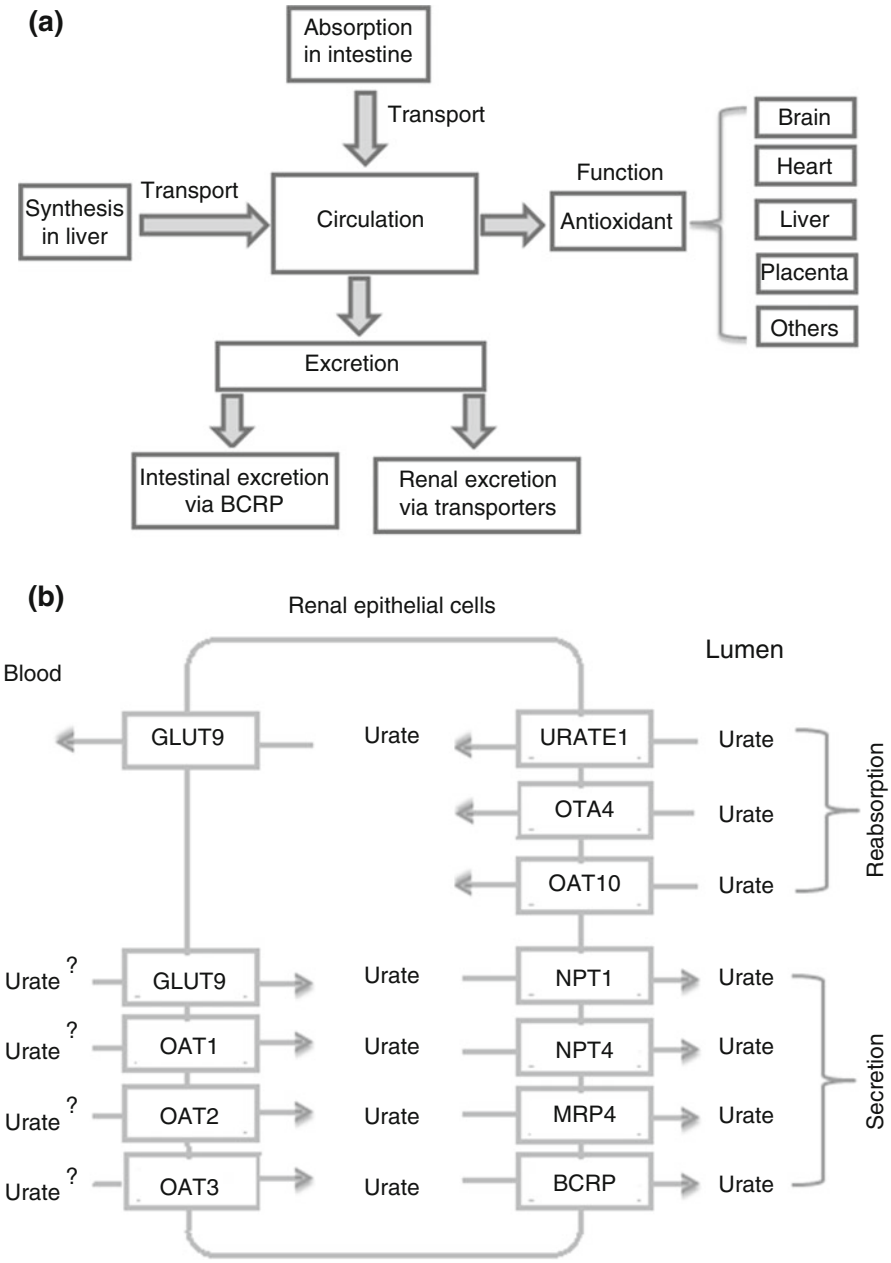

Fig. 1.3 (**a**) Diagram depicting the remote communications among organs mediating urate handling. Urate is either absorbed in the intestine or synthesized in the liver. Its absorption, distribution, and excretion are mediated by a battery of transporters in individual organs to achieve urate homeostasis in the cell, organ, and at the system level. (**b**) Urate transporters in the kidney which are involved in secretion and reabsorption of urate

OATP2A1 (Kanai et al. 1995). Other SLC transporters including OAT3 have been identified to transport PGs. Their tissue distribution is consistent with the fact that PGs are able to be removed locally to keep its low levels in circulation. For instance, during resting states, the circulating PGE2 is very low (Sánchez-Moreno et al. 2004). Upon cytokine stimulation or fever, the level of circulating PGE2 can increase up to fourfold. The increased levels of PGE2 also exist in cerebrospinal fluid, which is probably due to the entry of PGE2 into the cerebral ventricle from the peripheral circulation (Davidson et al. 2001). In the brain, PGE2 and PGD2 are involved in multiple brain pathophysiological processes, including modulation of synaptic plasticity, neuroinflammation, fever, and sleep promotion. Thus, concentrations of PGE2 and PGD2 in brain interstitial fluid and cerebrospinal fluid are maintained at appropriate levels for normal brain function, which is mainly attributed to efflux transporters on BBB and blood-cerebrospinal fluid barrier including OAT3, MRP4, and PG transporter (Akanuma et al. 2010; Tachikawa et al. 2014; Tachikawa et al. 2012a, b). Lipopolysaccharide was reported to decrease PGE2 elimination across BBB via downregulating OAT3 protein, becoming a reason inducing increases in levels of brain PGE2 (Akanuma et al. 2011). β-lactam antibiotics (such as cefazolin and ceftriaxone) significantly inhibited PGE2 elimination clearance from the CSF via inhibiting MRP4 function, leading to increase in the brain level of PGE_2 (Tachikawa et al. 2012a, b; Akanuma et al. 2010), which seem to explain clinical findings that some β-lactam antibiotics induce drug fever (Oizumi et al. 1989; Wilartratsami et al. 2014; Guleria et al. 2014).

A renal-specific PG transporter (OAT-PG) has been identified (Shiraya et al. 2010). OAT-PG, belonging to OAT family, recognizes PGs (such as PGH_2, PGB_2, PGD_2, PGE_1, PGE_2, $PGF_{1\alpha}$, and $PGF_{2\alpha}$). OAT-PG is localized to the basolateral membrane of proximal tubules and mediates transport of PGE_2 is into proximal tubule cells, where PGE can be rapidly metabolized to inactive metabolite 15-keto-PGE2 (Shiraya et al. 2010), inferring roles of OAT-PG in disposal of circulating PGE2.

Last example is chronic kidney disease. Chronic kidney disease is associated with the accumulation of small-molecule uremic toxins. These toxins are potentially toxic to cells and tissues via different mechanisms. More than 100 uremic toxins have been identified including indole derivatives, hippurate, kynurenine, polyamines, and other molecules. Some of them originate from gut microbiome and are then metabolized into more toxic compounds by hepatic drug-metabolizing enzymes. In addition to association with toxicity, some toxins also have important physiological activities. They serve as important metabolic intermediates, potential to affect redox state, influence cell growth, or act as ligands for signaling molecules in remote tissues. These toxins are often substrates or inhibitors of these drug transporters, inferring that these drug transporters are also implicated in disposal of uremic toxins (Nigam 2015). Clinical trial has demonstrated that metabolic alterations observed in diabetic kidney disease are associated with decreases in expression of renal OAT1 and OAT3. *Oat1* knockout mice express a similar pattern of reduced levels of urinary organic acids (Sharma et al. 2013). Downregulation of these transporter

function and expression further enhances accumulation of uremic toxins and worsens renal injury. On the hand, the increased levels of uremic toxins in circulation increase entry of uremic toxins into the brain or inhibit efflux of uremic toxins from the brain due to inhibition of transporter function, leading to accumulation of uremic toxins in the brain, which become reasons leading to uremic encephalopathy (Hosoya and Tachikawa 2011; Bugnicourt et al. 2013).

As described above, drug transporters mediate transport of not only therapeutic drugs but also endogenous compounds including signal molecules, later implicating in development and progression of some disease. Thus, drug transporters are also considered as novel targets for drug design and important factors for interpretation of inter-individual difference in response to drugs (Lin et al. 2015). Moreover, growing evidences have demonstrated drug toxicities related to interactions with drug transporters. Although as industry, regulatory agencies, and academic institutions, their focus are still on mechanisms of drug elimination and distribution, as well as drug-drug interactions through drug transporters, roles of drug transporters to endogenous biological activity attract increasingly people's eyes. Thus, a deeper understanding of these transporters in metabolic pathways, systems physiology, and morphogenesis will ultimately shed light on unexplained side effects of drugs and complex disease states.

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Chapter 2
ABC Family Transporters

Xiaodong Liu

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

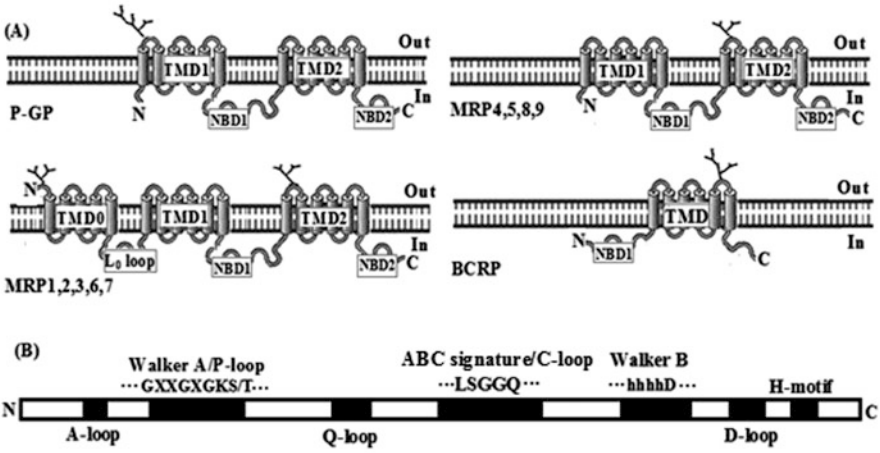

Fig. 2.1 (**a**) Secondary structure models of drug efflux transporters of the ATP-binding cassette family. TMD, transmembrane domain; NBD, nucleotide-binding domain. "In" and "out" are termed as cytoplasmic and extracellular compartments, respectively. (**b**) Linear arrangement and residues of the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

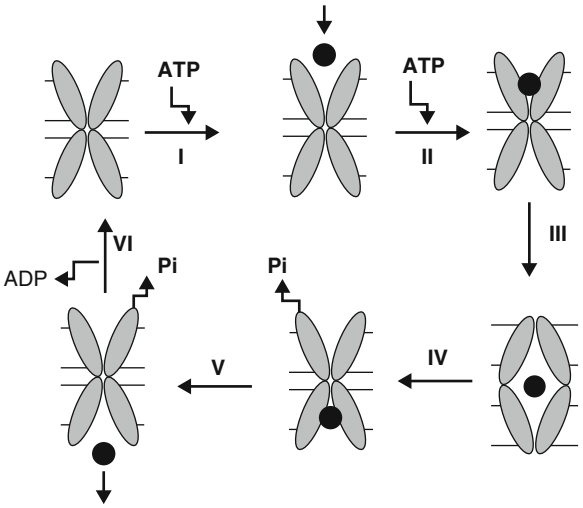

Fig. 2.2 Switch models for catalytic cycle of ATP binding and hydrolysis in the ABC transporter NBD dimer: Step I, resting state. The ATP-binding cassette monomers are separated in the absence of nucleotide. From Step I to Step II, the monomers are loaded processively with ATP, triggering the closure 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).

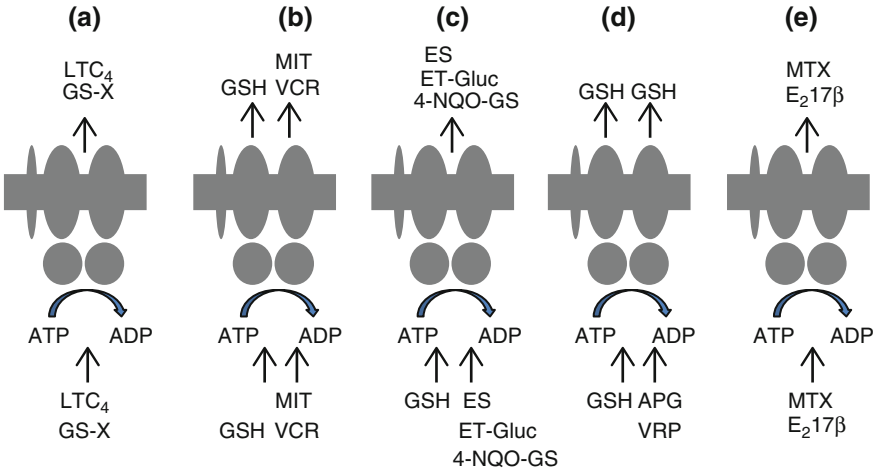

Fig. 2.3 Schematic representation of MRP1-mediated transport that involves GSH. (**a**) GS-conjugated transport, (**b**) GSH-drug cotransport, (**c**) GSH-stimulated conjugated transport, (**d**) drug-stimulated GSH transport, and (**e**) GSH-independent transport. *APG* apigenin, *ES* estrone sulfate, *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.

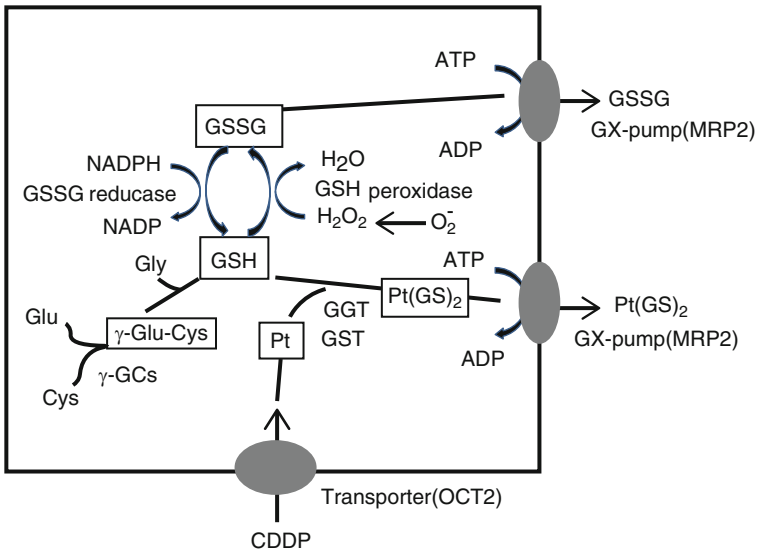

Fig. 2.4 The role of GSH and MRP2 in MRP-mediated CDDP (cisplatin) transport. *GST* glutathione-S-transferase, *GGT* γ-glutamyltranspeptidase, *Glu* glutamine, *Cys* cysteine, *Gly* glycine, *Pt (GS)2* platinum conjugates with GSH, *OCT2* organic cation transporter 2, *γ-GCs* γ-glutamylcysteine synthetase

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).

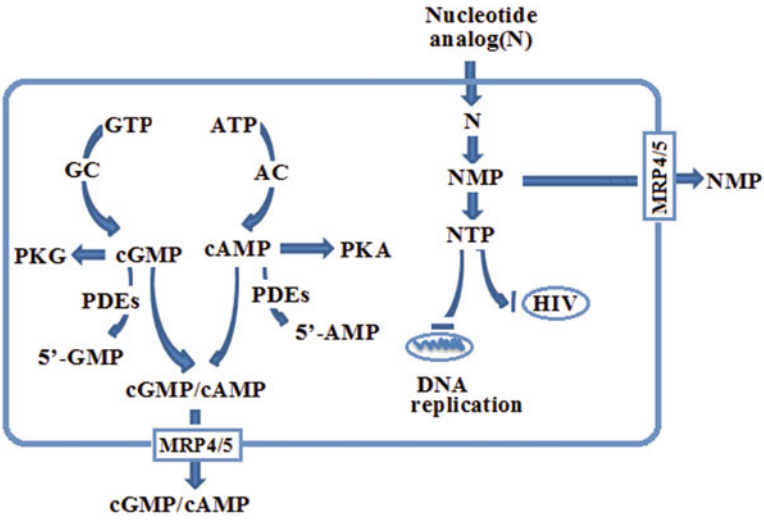

Fig. 2.5 Roles of MRP4 and MRP5 in cyclic nucleotide (cAMP/cGMP) signals and in resistance to nucleotide analogues (N). Overexpression of MRP4 or MRP5 can lower the intracellular concentrations of cAMP or cGMP, in turn, affecting the intracellular transduction of these mediators. Furthermore, monophosphate 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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Chapter 3
SLC Family Transporters

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Abstract Solute carrier (SLC) family transporters utilize an electrochemical potential difference or an ion gradient generated by primary active transporters for transporting their substrates across biological membranes. These transporters are categorized as facilitated transporters or secondary active transporters. More than 300 *SLC* transporters have been identified. *SLC* transporters related to drug transport mainly include *SLC21* gene subfamily (organic anion-transporting polypeptides, OATPs), *SLC22A* gene subfamily (organic anion transporters, OATs; organic cation transporters, OCTs; or organic cation/carnitine transporters, OCTNs), *SLC15A* gene subfamily (peptide transporters, PEPTs), and *SLC47A* gene subfamily (multidrug and toxin extrusion, MATEs). In general, OCTs transport organic cations, OATPs transport large and fairly hydrophobic organic anions, OATs transport the smaller and more hydrophilic organic anions, and PEPTs are responsible for the uptake of di-/tripeptides and peptide-like drugs. MATEs are responsible for efflux of organic cations. These transporters also transport some endogenous substances, indicating that the dysfunction of SLCs not only disrupts homeostasis but also largely impacts on the disposition of their substrate drugs. This chapter will discuss these SLC family transporters, with an emphasis on tissue distribution, substrate specificity, transporter physiology, and clinical significance.

Keywords Solute carrier · Organic anion-transporting polypeptides · Organic cation transporters · Organic cation/carnitine transporters · Organic anion transporters · Peptide transporters · Multidrug and toxin extrusion

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3.1 General Introduction

Drug disposition is usually involved in drug transporters which are classified as influx and efflux transporters. Efflux transporters, principally the ABC transporters, are responsible for moving drugs out of cells, and influx transporters, primarily the solute carrier (SLC) transporters, mediate drug uptake into cells. The SLC transporters utilize an electrochemical potential difference or an ion gradient generated by primary active transporters for transporting their substrates across biological membranes; thus, SLC transporters are categorized as facilitated transporters or secondary active transporters. More than 300 SLC transporters have been identified. They are expressed in key tissues such as the kidney, liver, intestine, and brain, playing crucial roles in maintaining body homeostasis. Among the SLC transporters, *SLC21* gene subfamily (organic anion-transporting polypeptides, OATPs), *SLC22A* gene subfamily (organic anion transporters, OATs; organic cation transporters, OCTs; or organic cation/carnitine transporters, OCTNs), *SLC15A* gene subfamily (peptide transporters, PEPTs), and *SLC47A* gene subfamily (multidrug and toxin extrusion, MATEs) are considered to play a key role in disposition of therapeutic agents. Although MATEs belong to SLC family transporters, they act as efflux transporters. In general, OCTs transport organic cations, OATPs transport large and fairly hydrophobic organic anions, OATs transport the smaller and more hydrophilic organic anions, and PEPTs are responsible for the uptake of di-/tripeptides and peptide-like drugs. MATEs mediate efflux of organic cations. Their substrates include both endogenous and exogenous substances, indicating that the dysfunction of SLCs not only disrupts homeostasis but also largely impacts on the disposition of their substrate drugs. This chapter will discuss SLC family transporters, with an emphasis on tissue distribution, substrate specificity, and transporter physiology.

3.2 Organic Anion-Transporting Polypeptides (OATPs)

3.2.1 General Characteristics of OATPs

Organic anion-transporting polypeptides (OATPs for human and Oatps for other species) are coded by *SLC21/SLCO* genes according to the nomenclature of the HUGO Gene Nomenclature Committee (Hagenbuch and Meier 2004). Up to date, 11 human OATP transporters have been identified. Based on phylogenetic relationships and chronology of identification, OATPs have been divided into families designated by an Arabic numeral. The general rules for this classification system are that proteins with more than 40% of amino acid sequence identity belong to the same family designated by Arabic numbering, while proteins with more than 60% of amino acid sequence identity belong to the same subfamily designated by letters. If there are several individual gene products (proteins) within the same subfamily, additional continuous Arabic numbering based on the chronology of identification

Table 3.1 Human OATP transporters and their rodent orthologues. (Hagenbuch and Meier 2003, 2004; Hagenbuch and Stieger 2013; Tamai 2012)

Human protein	Old name	Human gene	Rodent gene	Rodent protein	Old name	Similarities to human (%)
OATP1A2	OATP-A	*SLCO1A2*	*Slco1a1*	Oatp1a1	Oatp1	67
			Slco1a3(*rats*)	Oatp1a3	Oat-k1, Oat-k2	66
			Slco1a4	Oatp1a4	Oatp2	73
			Slco1a5	Oatp1a5	Oatp3	72
			Slco1a6	Oatp1a6	Oatp5	66
OATP1B1	OATP-C, LST-1	*SLCO1B1*	*Slco1b2*	Oatp1b2	Oatp4	64
OATP1B3	OATP8, LST-2	*SLCO1B3*	*Slco1b2*	Oatp1b2	Oatp4	66
OATP1C1	OATP-F	*SLCO1C1*	*Slco1c1*	Oatp1c1	Oatp14	85
OATP2A1	PGT	*SLCO2A1*	*Slco2a1*	Oatp2a1	PGT	82
OATP2B1	OATP-B	*SLCO2B1*	*Slco2b1*	Oatp2b1	Oatp9	77
OATP3A1	OATP-D	*SLCO3A1*	*Slco3a1*	Oatp3a1	Oatp11	97
OATP4A1	OATP-E	*SLCO4A1*	*Slco4a1*	Oatp4a1	Oatp12, OatpE	76
OATP4C1	OATP-H	*SLCO4C1*	*Slco4c1*	Oatp4c1	Oatp-H	No data
OATP5A1	OATP-J	*SLCO5A1*	/	/	/	/
OATP6A1	OATP-I	*SLCO6A1*	*Slco6b1*	Oatp6b1	Gst-1	No data
			Slco6c1	Oatp6c1	Gst-2	No data
			Slco6d1	Oatp6d1	Oatp17	No data

should be used (Hagenbuch and Meier 2004). On this basis, the human OATPs are classified into six families (OATP1, OATP2, OATP3, OATP4, OATP5, and OATP6), and each family can have several families (such as OATP1A, OATP1B, and OATP1C). Within these subfamilies, the individual OATPs are numbered according to the chronology of their identification, and if there is already an orthologue known, they are given the same number. Thus, the human OATPs are termed as OATP1A2, OATP1B1, OATP1B3, OATP1C1, OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP5A1, and OATP6A1, respectively (Table 3.1).

A significant amount of gene duplication and divergence for this family has occurred in animals, especially rodents. OATP1A2 has five rodent orthologues: Oatp1a1, Oatp1a3 (in rats only), Oatp1a4, Oatp1a5, and Oatp1a6. OATP1B1 and OATP1B3 share a single rodent orthologue, Oatp1b2. The other OATPs and their rodent orthologues are OATP1C1 (Oatp1c1), OATP2A1 (Oatp2a1), OATP2B1 (Oatp2b1), OATP3A1 (Oatp3a1), OATP4A1 (Oatp4a1), OATP4C1 (Oatp4c1), OATP5A1, and OATP6A1 (Oatp6b1, Oatp6c1, and Oatp6d1) (Roth et al. 2012). There exist large interspecies difference for function and expression of OATPs between animals and humans. For example, rat Oatp1b2 is expressed in the liver, and its similarities in amino acid sequences with human OATP1B1 (64%) and OATP1B3 (66%) are equivalent, but a contribution of OATP1B3 and Oatp1a4 to

transport of antibiotics is largest in the liver of human and rat (Nakakariya et al. 2008), respectively. However, the rat Oatp1a4 only shares 45% amino acid sequence identity with human OATP1B3, demonstrating that amino acid sequence similarity may not be adequate to correlate human OATPs and rat Oatps functionally (Tamai 2012). In general, SLCO family members are poorly conserved evolutionarily; some orthologues of Oatps expressed in rodents do not exist in human. Oatp1a1 and Oatp1a4 are expressed in the liver of rodents and contribute to the hepatic uptake of their substrates, but their orthologues in humans have not been identified. In human, OATP1B1 and OATP1B3 play key roles in the hepatic uptake of drugs; their characteristics are quite different from those of Oatp1a1 and Oatp1a4. In addition, dogs have only one Oatp1b4 (Niemi et al. 2011). In mouse, Oatp1a4 is expressed in both luminal and abluminal membrane of brain capillary endothelial cells. In human, OATP1A2 is only localized to the luminal side of brain capillary endothelial cells (Shitara et al. 2013a). These results indicate that the extrapolation of OATP roles in drug disposition from animals to humans may be difficult.

OATPs share a 12-transmembrane domain (TMD) structure with superfamily signature at the extracellular border of TMD6, the large extracellular loop between TMD9 and 10, and multiple N-glycosylation sites in the extracellular loops 2 and 5 (Meier-Abt et al. 2005; Zhou et al. 2017) (Fig. 3.1). Their superfamily signature D-XRW-(I,V)-GAWW-X-G-(F,L)-L consists of 13 amino acids, which are well conserved between human OATPs and rodent Oatps. The large extracellular region between TMD9 and 10 contains 11 conserved cysteine residues, all of which are disulfide bonded. Mutation of just one cysteine residue can destroy OATP function, indicating a central role of the large extracellular region in transport of substances (Meier-Abt et al. 2005). The N-glycosylation also impacts on OATP function (Zhou et al. 2017).

Most OATPs transport a wide spectrum of amphipathic organic compounds. In general, OATP substrates are anionic amphipathic molecules with a rather high molecular weight (>350Da) and a high degree of albumin binding under physiological

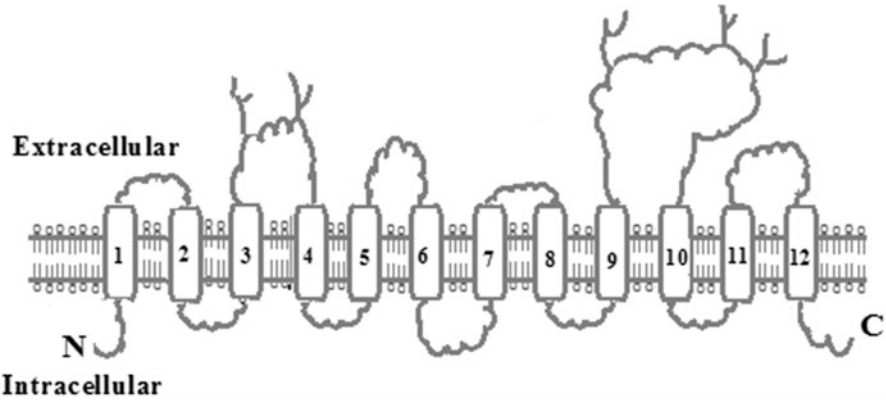

Fig. 3.1 Schematic representation of the secondary structure of OATPs. Transmembrane segments (TMDs) are numbered from 1 to 12. "Y" shapes represent potential glycosylation sites

conditions. OATPs contain two hydrogen bond acceptors, one hydrogen bond donor, and two hydrophobic regions, which are met by most OATP substrates including bile salts, steroids, and some cyclic/linear peptides (Hagenbuch and Meier 2004). OATPs mediate transport of certain endogenous compounds including bile acids, thyroid hormones, prostaglandins, eicosanoids, steroids(/and their conjugates). OATPs also accept a number of therapeutic agents as their substrates such as statins, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, antibiotics, antihistaminics, and anticancer drugs. Interestingly, OATP substrates are often substrates of some ABC transporters such as multidrug resistance-associated proteins (MRPs), breast cancer resistance protein (BCRP), or P-glycoprotein (P-GP); thus their efficient transport of substrate is realized by the cooperative functions of OATP-mediated uptake and ABC-mediated efflux. Moreover, most of them are often metabolized by enzymes (such as CYP450 and conjugating enzymes) in the liver. Thus, hepatic uptake transporters (such as OATPs), drug metabolism enzymes, and ABC transporters work in concert to enable the hepatobiliary elimination of anionic drugs which is called as "drug transporter-metabolism interplay" (Salphati 2009) or "drug transporter-metabolism alliance" (Benet 2009). The drug transporter-metabolism interplay also exists in other tissues such as the intestine (Benet 2009) and kidney. The existence of drug transporter-metabolism interplay will partly explain how genetic variants of drug transporters and enzymes influence the inter-individual variability of drug elimination (Nies et al. 2008).

Although exact common transport mechanism of OATPs is not established, transport of OATPs has been suggested to occur through a central, positively charged pore in a so-called rocker-switch type of mechanism (Fig. 3.2). In this mechanism, the transport protein is assumed to have two major alternating conformations: inward-facing (C_i) and outward-facing (C_o). At any moment, a single-

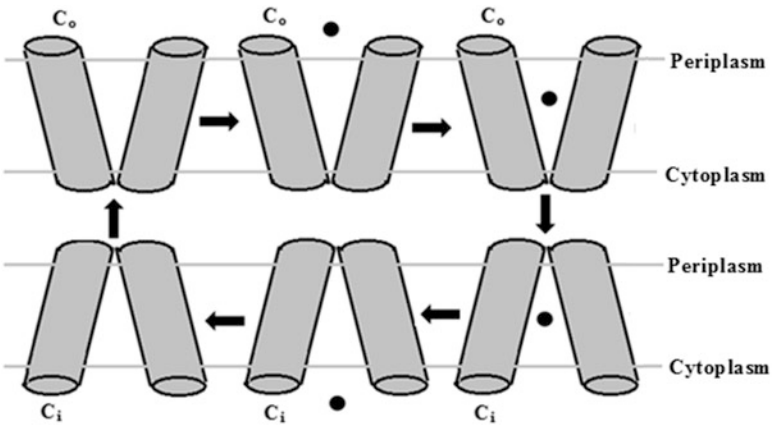

Fig. 3.2 Schematic diagram of the single-binding site, alternating access mechanism with a rocker-switch type of movement for the substrate transport reaction. The diagram describes the proposed conformational changes that the transporter undergoes during the reaction cycle. C_o represents the protein in the outward-facing conformation and C_i the inward-facing one. Point represents substrate

binding site in a pore is accessible from only one side of the membrane. Interconversion between the two conformations is only possible via a substrate-bound form of the transport protein (Meier-Abt et al. 2005).

OATP-mediated transport is generally independent of sodium, chloride, and potassium gradients, membrane potential, and ATP levels. OATPs are also considered to be electroneutral exchangers, in which the cellular uptake of organic anions is coupled to the efflux of another anion such as HCO_3^-, GSH, and/or glutathione-S conjugates (Roth et al. 2012). However, the exchange mechanism has been supported only by rat Oatp1a1 (Li et al. 1998; Satlin et al. 1997). Thus real energy coupling transport mechanisms of human OATPs remain poorly understood. Several studies have demonstrated that different OATPs show different transport mechanisms. In *Xenopus laevis* oocytes expressing OATP1B1 or OATP1B3, uptake of taurocholate mediated by OATP1B1 or OATP1B3 was cis-inhibited by taurocholate and estrone-3-sulfate (E3S), not GSH. Likewise, transport of [^{3}H]taurocholate and [^{3}H]E3S were trans-stimulated by E3S and taurocholate, not GSH. In addition, OATP1B3 mediated neither GSH efflux nor GSH-taurocholate cotransport out of cells. These results indicate that OATP1B3 and OATP1B1 most likely function as bidirectional facilitated diffusion transporters and that GSH is not a substrate or activator of their transport (Mahagita et al. 2007).

OATP-mediated organic anion exchange seems to be pH-dependent and electroneutral. However, these results are often confusing. For example, OATP2B1 transport is enhanced by acidic pH, and a proton gradient is considered to be a driving force for OATP2B1 (Nozawa et al. 2004; Sai et al. 2006; Kis et al. 2010). The pH dependency of OATPs may be linked to a highly conserved His in TMD3 (Leuthold et al. 2009). OATP1C1 lacks a highly conserved histidine in TMD3, and its transport is insensitive to pH. But, mutant of Gln130 with His in OATP1C1 acquired pH sensitivity of substrate uptake. On the contrast, mutant of His107 with Gln in Oatp1a1 abolished pH sensitivity of substrate transport. In human embryonic kidney 293 (HEK293) cells expressing OATP2B1, it was showed that uptake activities for different compounds have different pH sensitivities (Nozawa et al. 2004). For example, dehydroepiandrosterone sulfate (DHEAS), E3S, and fexofenadine were transported by OATP2B1 at both neutral and acidic pH, whereas estradiol-17β-D-glucuronide ($E_2$17βG) was not transported at all. Transport of taurocholic acid and pravastatin by OATP2B1 occurred only at acidic pH. OATP1B3- or OATP1B1-mediated E3S uptake was unaffected by depolarization of the membrane potential or pH (Mahagita et al. 2007). The characteristics of rat Oatp1a1-, OATP1B1-, and OATP1B3-mediated transport of taurocholic acid and $E_2$17βG are also different (Martinez-Becerra et al. 2011). Extracellular pH acidification stimulated Oatp1a1- and OATP1B3-mediated uptakes of taurocholic acid and $E_2$17βG, but inhibited OATP1B1-mediated uptakes of taurocholic acid and $E_2$17βG. Extracellular pH acidification enhanced Oatp1a1- and OATP1B3-mediated E3S uptake without affecting OATP1B1-mediated E3S uptake. Oatp1a1 is an electroneutral anion exchanger, but transports mediated by OATP1B1 and OATP1B3 are electrogenic. Moreover, OATP1B1- and OATP1B3-mediated transports of taurocholic acid, $E_2$17βG and E3S were also associated to the net anion influx (Martinez-Becerra et al. 2011).

3.2.2 OATP1B1

3.2.2.1 General Properties and Tissue Distribution of OATP1B1

OATP1B1 (previously named as OATP2, OATP-C, and LST-1), a 691-amino acid glycoprotein coded by *SLCO1B1* gene, is mainly expressed on the sinusoidal membrane of human hepatocytes. Its apparent molecular mass is 84 kDa, which is reduced after deglycosylation to 58 kDa (Niemi et al. 2011). Although SLCO1B1 mRNA has been also detected in other tissues including intestinal enterocytes, OATP1B1 protein is mainly localized to the basolateral membrane of hepatocytes. Several studies have demonstrated that OATP1B1 is also expressed in hormone-independent breast cancer cells (MDA/LCC6-435 and MDA-MB-231) (Banerjee et al. 2012) and ovarian carcinoma cell lines (SK-OV-3) (Svoboda et al. 2011). High expression of OATP1B1 was also detected in some cancer tissues (such as colon polyps, colon cancer, and ovarian cancer) derived from nonhepatic tissues which normally do not express OATP1B1 (Ballestero et al. 2006; Svoboda et al. 2011).

OATP1B1 shares 80% amino acid sequence identity with OATP1B3 (Tamai 2012). In general, orthologues for human OATP1B1 may not exist in rodents. Rodent Oatp1b2 is considered to be the closest orthologue of OATB1B1 and OATP1B3 (Tamai 2012). Like most OATPs, OATP1B1 has 12 TMDs and a highly conserved signature sequence. Roles of these conserved amino acids in function and expression were confirmed using site-directed mutagenesis (Gui and Hagenbuch 2009; Huang et al. 2013; Taylor-Wells and Meredith 2014; Weaver and Hagenbuch 2010). For example, simultaneous mutations of Leu545, Phe546, Leu550, and Ser554 caused almost complete loss of OATP1B1-mediated E3S transport (Gui and Hagenbuch 2009). Mutant Trp258 with alanine had only one substrate-binding site that altered its capability to interact with E3S but retained the ability to transport taurocholate. Mutant Trp259 retained the biphasic characteristic of the transporter for E3S, but showed great reduction in transport function of taurocholate, whose K_m value was more higher eightfold increase than that of the wild type or Trp258A (Huang et al. 2013). Similarly, substitution of Asp251 with glutamic residue, Trp254 with phenylalanine, or Trp258/Trp259 with phenylalanine significantly decreased transport of E3S and surface expression of OATP1B1 proteins (Taylor-Wells and Meredith 2014).

3.2.2.2 Substrates and Inhibitors of OATP1B1

A series of endogenous substrates of OATP1B1 including bilirubin/unconjugated bilirubin, thyroid hormones, eicosanoids, DHEAS, $E_2$17βG, and E3S have been identified using transiently or stably OATP1B1-expressing cell lines. Bile acids and their conjugates are endogenous substrates of OATP1B1, but conjugated bile acids are preferred to unconjugated bile acids as substrates for OATP1B1 (Suga et al. 2017). Coproporphyrins I and III are also endogenous substrates of OATP1B1 and

Table 3.2 Substrates of several human OATP1B1

Endogenous substrates	
Bile acids	Cholic acid, glycocholic acid, glycoursodeoxycholic acid, taurolithocholic acid 3-sulfate, taurocholic acid, tauroursodeoxycholic acid
Eicosanoids	Leukotriene C4, leukotriene E4, prostaglandin E2, thromboxane B
Thyroid hormones	Thyroxine, triiodothyronine, iodothyronine sulfates
Steroids	DHEAS, $E_2$17βG, E3S
Bilirubins	Bilirubin, bilirubin monoglucuronide, bilirubin diglucuronide
Others	[D-Ala^2,D-Leu^5]-enkephalin, coproporphyrins I, coproporphyrins III
Therapeutic drugs	
Stains	Atorvastatin, cerivastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin
Antibacterials	Benzylpenicillin, caspofungin, cefditoren, cefoperazone, cefazolin, erythromycin, nafcillin, rifampin
Anticancer drugs	Gimatecan, SN-38, docetaxel, paclitaxel, regorafenib, sorafenib, methotrexate
HIV protease inhibitors	Saquinavir, lopinavir, darunavir
Antihypertensive agents	Atrasentan, bosentan, enalapril, olmesartan, temocapril, valsartan
Antidiabetic drugs	Glibenclamide, nateglinide, repaglinide, troglitazone sulfate
Other drugs	Mycophenolic acid-O-glucuronide, sirolimus, torasemide, flavopiridol
Diagnostic markers	Bromosulfophthalein, fluorescein, cholyl-glycylamido-fluorescein 8-fluorescein, fluorescein-methotrexate
Toxins	Arsenate, arsenite, demethylphalloidin, phalloidin

can be served as functional markers of OATP1B activity (Bednarczyk and Boiselle 2016; Shen et al. 2016). OATP1B1 also accepts several therapeutic drugs as substrates (Table 3.2).

The first identified substrate drug for OATP1B1 is pravastatin. Now, all statins in clinical use are substrates of OATP1B1. Other substrate drugs of OATP1B1 include angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists, diuretics, endothelin receptor antagonists, antibiotics, HIV protease inhibitors, anticancer agents, antidiabetic drugs (glibenclamide, nateglinide, and repaglinide), anti-inflammatory drugs, and antihistamine (Niemi et al. 2011; Ohya et al. 2015; Zimmerman et al. 2013). Some diagnostic markers or fluorescent dyes such as gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid, sodium fluorescein, and bromosulfophthalein (BSP) are also OATP1B1 substrates (Bruyn et al. 2011; Niemi et al. 2011). It should be noted that substrate specificity of OATP1B1 commonly overlaps that of other OATPs including OATP1B3 and OATP2B1 (Niemi et al. 2011). For example, fluvastatin, rosuvastatin, $E_2$17βG, pitavastatin, rifampicin, olmesartan, valsartan, atrasentan, and bosentan are bisubstrates of both OATP1B1 and OATP1B3. Pravastatin and atorvastatin are also substrates of OATP2B1. But, substrate specificities of OATP1B1 and OATP1B3 have been identified. For instance, E3S can be preferentially recognized by OATP1B1 rather

than OATP1B3, whereas cholecystokinin octapeptide (CCK-8) and telmisartan are specifically transported by OATP1B3 not OATP1B1 (Ishiguro et al. 2006; Maeda 2015; Niemi et al. 2011). However, reports whether paclitaxel and docetaxel are substrates of OATP1B1 remain controversial (de Graan et al. 2012; Iusuf et al. 2015; Lee et al. 2015; Nieuweboer et al. 2014). Interaction of paclitaxel with human OATP1B1 and OATP1B3 seems to be dependent on cell types. Both OATP1B1 and OATP1B3 expressed in HEK293 cells or Chinese hamster ovary (CHO) cells mediated transport of docetaxel, but in *Xenopus laevis* oocytes, OATP1B3 not OATP1B1 mediated transport of docetaxel (Nieuweboer et al. 2014). However, in HeLa cells, OATP1A2, OATP1B1, OATP1B3, and OATP1C1 all mediated uptake of docetaxel (Lee et al. 2015). These results indicate that most experiments to elucidate the mechanism of action of OATPs have been conducted in *Xenopus laevis* oocytes, but the findings cannot be excluded entirely that these transporters behave differently in the context of a mammalian cell membrane. In addition, the OATP1B1-medaited transports of E3S and 17α-ethinylestradiol sulfate show biphasic kinetics, suggesting the possibility of multiple substrate-binding sites in OATP1B1 (Han et al. 2010; Noé et al. 2007).

A series of OATP1B1 inhibitors have been identified including atazanavir, atorvastatin, BSP, caspofungin, clarithromycin, clotrimazole, cyclosporine, digoxin, $E_2$17βG, eltrombopag, erythromycin, E3S, gemfibrozil, gemfibrozil-1-O-glucuronide, glyburide, hyperforin, indinavir, irinotecan, ketoconazole, lovastatin, metyrapone, mifepristone, nelfinavir, nilotinib, paclitaxel, pazopanib, pioglitazone, pravastatin, repaglinide, rifampicin, rifamycin SV, ritonavir, rosiglitazone, roxithromycin, saquinavir, sildenafil, simvastatin, SN-38, sorafenib, tacrolimus, taurocholic acid, telmisartan, tipranavir, troglitazone, troglitazone sulfate, and valsartan (Allred et al. 2011; Hu et al. 2014a; Izumi et al. 2013; Kalliokoski and Niemi 2009; Xu et al. 2013). Flavonoids (such as biochanin A, epigallocatechin-3-gallate, and genistein) (Wang et al. 2005) and catechins (such as epicatechin gallate and epigallocatechin gallate) (Roth et al. 2011) were also identified to be OATP1B1 inhibitors.

A method for classification of OATP1B1 inhibitors was proposed (Kotsampasakou et al. 2015), whose threshold inhibitory concentration (IC_{50}) value of OATP1B1 inhibitor was set to be 10 μM. Drugs with IC_{50} values less than 1 μM are considered as strong inhibitors, compounds with IC_{50} values between 1 and 5 μM as moderately strong inhibitors, compounds with IC_{50} values between 5 and 10 μM as moderate inhibitors, and compounds having IC_{50} values above 10 μM as slight inhibitors, and drugs whose IC_{50} value is impossible to obtain are considered as noninhibitor. Thus, asunaprevir, atorvastatin, bromocriptine, BSP, carfilzomib, cyclosporine A, dronedarone, E3S, estropipate, glyburide, hyperforin, indocyanine green, lapatinib, lopinavir, nelfinavir, paclitaxel, rifampin, rifamycin SV, ritonavir, simeprevir, sirolimus, tacrolimus, and telmisartan are strong OATP1B1 inhibitors, whose K_i or IC_{50} values are below 1 μM for at least a substrate (Furihata et al. 2014; Izumi et al. 2013; Kotsampasakou et al. 2015; Kalliokoski and Niemi 2009; Niemi et al. 2011).

It is noteworthy that inhibition of OATP1B1 is often substrate-dependent. Izumi et al. (2013) reported substrate-dependent inhibitory effect on uptake of $E_2$17βG, E3S, and BSP in CHO cells expressing OATP1B1 using E3S, cyclosporine A, BSP, ritonavir, rifampin, tacrolimus, erythromycin, $E_2$17βG, ketoconazole, taurocholate, verapamil, gemfibrozil, or probenecid as OATP1B1 inhibitors. They found that $E_2$17βG was the most sensitive to the tested inhibitors, exhibiting the lowest IC_{50} values for all the tested OATP1B1 inhibitors. Ritonavir, gemfibrozil, erythromycin, and rifampin caused remarkably substrate-dependent inhibition. The IC_{50} values (μM) of ritonavir (0.397, 46.4, and 3.38 for $E_2$17βG, E3S, and BSP uptake, respectively), gemfibrozil (26.4, 381, and 173, respectively), erythromycin (4.88, 13.4, and 63.3, respectively), and rifampin (0.585, 6.96, and 2.75, respectively) showed up to 117-, 14-, 13-, and 12-fold variation depending on the substrates, respectively. Based on above Kotsampasakou et al. (2015) definition, ritonavir is strong inhibitors for $E_2$17βG, slight inhibitors for E3S, and moderately strong inhibitors for BSP, respectively. Similarly, substrate-dependent inhibitory effects of cyclosporine A, rifampin, and gemfibrozil on OATP1B1-mediated transport of 12 clinically used substrate drugs (pitavastatin, atorvastatin, fluvastatin, rosuvastatin, pravastatin, repaglinide, nateglinide, glibenclamide, bosentan, valsartan, torasemide, and fexofenadine) were also observed in HEK293 cells expressing OATP1B1 (Izumi et al. 2015). The results showed that K_i values (μM) for cyclosporine A varied from 0.0771 (fexofenadine) to 0.486 (torasemide) (6.3-fold variability), for rifampin from 0.358 (nateglinide) to 1.23 (torasemide) (3.4-fold variability), and for gemfibrozil from 9.65 (pravastatin) to 252 (nateglinide) (26-fold variability), respectively. Except for the inhibition of torasemide uptake by cyclosporine A and that of nateglinide uptake by gemfibrozil, the K_i values were within 2.8-fold of those obtained using $E_2$17βG as a substrate. However, E3S and BSP yielded the higher K_i values of all three inhibitors compared with the clinically used substrate drugs. Therefore, $E_2$17βG seems to behave similarly to the clinically used substrate drugs in terms of the susceptibility to OATP1B1 inhibition.

Gemfibrozil was reported to inhibit OATP1B1-mediated transport of fluvastatin, pravastatin, simvastatin, and taurocholate without affecting OATP-mediated transport of E3S and troglitazone sulfate (Noé et al. 2007). OATP1B1-mediated transport of E3S displayed biphasic saturation kinetics; gemfibrozil only inhibited the high-affinity component of E3S to OATP1B1. Importantly, although recombinant OATP1B1-, OATP2B1-, and OATP1B3-mediated fluvastatin transport was inhibited to 97, 70, and 62% by gemfibrozil (200 μM), respectively, fluvastatin uptake in primary human hepatocytes was only slight inhibited by gemfibrozil (200 μM) (27% inhibition), indicating that the in vitro systems are always not able to predict the behavior in more complex systems such as primary hepatocytes or in vivo results. Moreover, diclofenac (50 μM), ibuprofen (10 and 50 μM), and lumiracoxib (50 μM) inhibited BSP uptake, but stimulated pravastatin uptake (Kindla et al. 2011). These results indicate that OATP1B1-mediated drug-drug interaction risk can be seriously underestimated or overestimated if the substrate is not appropriately selected. Therefore, selection of substrate, substrate concentration, and in vitro transport system are critical for the conduct of in vitro interaction studies involving individual liver OATP carriers.

Several reports (Amundsen et al. 2010; Shitara et al. 2009; Shitara et al. 2012) have demonstrated that preincubation with cyclosporine A may potentiate inhibitory effect of cyclosporine A on OATP1B1 function. For example, inhibitory effect of cyclosporine A on OATP1B1-mediated uptake of atorvastatin following 1 h preincubation was approximately 22-fold more efficiently than coincubation of atorvastatin with cyclosporine A (Amundsen et al. 2010). In line with this, 30 min preincubation with 1 μM cyclosporine A also showed stronger inhibitory effects on E3S transport compared with coincubation of cyclosporine A and E3S without altering expression and intracellular localization of OATP1B1. Interestingly, the inhibitory effect lasted for at least 18 h after cyclosporine A removal. The long-lasting inhibition by cyclosporine A was also observed in human hepatocytes (Shitara et al. 2012). An in vivo study (Shitara et al. 2009) showed that 24 h after the subcutaneous administration of cyclosporine A to rats, the hepatic clearance of BSP was still significantly decreased, but expression of Oatp1a1 and Oatp1b2 as well as the amount of intrahepatic glutathione was little altered. Similar long-lasting inhibition on pravastatin transport by cyclosporine A was demonstrated in rats (Taguchi et al. 2016). Other drugs such as saquinavir, ritonavir, simeprevir, and asunaprevir also exhibited the long-lasting inhibition (Furihata et al. 2014; Shitara et al. 2013b), but the enhanced extents were less than cyclosporine A. The long-lasting inhibition suggests that a more complex mechanism exists and these agents show potently inhibitory effect on the transporter even at low concentrations.

It should be recognized that many OATP1B1 inhibitors are also potent inhibitors of other transporters or drug-metabolizing enzymes. For example, Karlgren et al. (2012) investigated effects of 225 compounds on functions of OATP1B1, OATP1B3, and OATP2B1. Ninety-one of the 225 investigated compounds were identified to interact with one or more of the three OATP transporters. Among them, 27, 3, and 9 compounds were identified specific inhibitors of OATP1B1, OATP1B3, and OATP2B1, respectively. Twenty-six compounds were identified as common inhibitors of 3 OATPs, 16 compounds were common inhibitors of OATP1B1 and OATP1B3, 9 compounds were common inhibitors of OATP1B1 and OATP2B1, and only nefazodone was identified as an inhibitor of both OATP1B3 and OATP2B1. A method for classification of OATP inhibitors (Karlgren et al. 2012) was proposed. The compound is considered to as specific inhibitor if it has a greater than tenfold lower IC_{50} value as compared to those of the other two OATPs. For example, IC_{50} values of pravastatin were 3.6 μM for OATP1B1, 62 μM for OATP1B3, and 190 μM for OATP2B1, respectively. And IC_{50} values of erlotinib were 21 μM for OAT1B1, 44 μM for OATP1B3, and 0.55 μM for OATP2B1, respectively. Thus, pravastatin and erlotinib are classified as selective inhibitors of OATP1B1 and OATP2B1, respectively. IC_{50} values of cyclosporine A were 1.4 μM for OATP1B1, 1.3 μM for OATP1B3, and 37 μM for OATP2B1, respectively. And IC_{50} values of rifampicin were 1.2 μM for OATP1B1, 1.5 μM for OATP1B3, and 65 μM for OATP2B1, respectively. The two compounds are identified as common inhibitors of OATP1B1 and OATP1B3. In addition, cyclosporine A is an inhibitor of the P-GP, OATP1B3, OATP2B1, BCRP, MRP2, and CYP3A4.

3.2.2.3 Pharmacological and Physiological Function of OATP1B1

OATP1B1 is almost exclusively expressed in human hepatocytes. Although other OATPs such as OATP1B3 and 2B1 are also expressed in the liver, but their expression in the liver is less than that of OATP1B1, inferring that OATP1B1 is the most important transporter (Hirano et al. 2004; Karlgren et al. 2012; Vildhede et al. 2014; Kunze et al. 2014). OATP1B1 accepts widely therapeutic drugs, as its substrates, suggesting that OATP1B1 plays a crucial role in the hepatic uptake and clearance of these therapeutic drugs.

In the liver, at first, drugs in blood are taken up into hepatocytes via both passive diffusion and transporter-mediated transport across sinusoidal membrane. Then, the drugs in hepatocytes are metabolized by metabolic enzymes, excreted into bile via transporter-mediated transport, and exported to blood via passive diffusion or transporter-mediated active transport (Fig. 3.3). Thus, the overall hepatic intrinsic clearance ($CL_{\text{int,all}}$) is expressed as a hybrid parameter consisting of membrane permeation clearances of unbound parent drugs for influx ($CL_{\text{int,up}}$) and efflux ($CL_{\text{int,back}}$) across the sinusoidal membrane, the intrinsic clearance for metabolism ($CL_{\text{int,met}}$), and biliary excretion of unbound drugs ($CL_{\text{int,bile}}$) (Eq. 3.1).

$$CL_{\text{int,all}} = CL_{\text{int,up}} \times \frac{CL_{\text{int,bile}} + CL_{\text{int,met}}}{CL_{\text{int,bile}} + CL_{\text{int,met}} + CL_{\text{int,back}}} \tag{3.1}$$

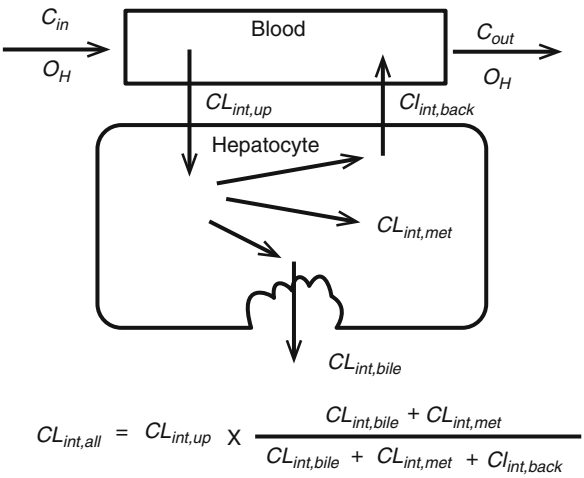

Fig. 3.3 Schematic model representing the elimination of drugs in the liver. Drugs are taken up into the liver across the sinusoidal membrane. Then some molecules are metabolized or excreted into bile or exported to blood. Symbol: $CL_{\text{int,all}}$, overall hepatic intrinsic clearance; $CL_{\text{int,up}}$, intrinsic uptake clearance; $CL_{\text{int,back}}$, intrinsic clearance of backflux to blood; $CL_{\text{int,met}}$, intrinsic metabolism clearances; $CL_{\text{int,bile}}$, biliary clearance of unbound drug; Q_H, hepatic blood flow. C_{in} and C_{out} concentrations of drug in arterial and venous blood, respectively

If sum of $CL_{int,bile}$ and $CL_{int,met}$ is much larger than $CL_{int,back}$, $CL_{int,all}$ becomes close to $CL_{int,up}$. That is to say that overall hepatic intrinsic clearance can be described by only uptake clearance, inferring that the transporter-mediated hepatic uptake is the limited step of hepatic clearance. Under the situation, although the drugs are extensively metabolized by metabolic enzymes, but in vitro metabolic clearance is not a good predictor of hepatic clearance. Therefore hepatic uptake clearance estimated using human hepatocytes should be introduced for accurate prediction.

On the other hand, if $CL_{int,back}$ is much larger than sum of $CL_{int,bile}$ and $CL_{int,met}$, $CL_{int,all} = CL_{int,up} \times (CL_{int,bile} + CL_{int,met})/CL_{int,back}$. Therefore, the value of $CL_{int,all}$ is determined by $CL_{int,up}$, $CL_{int,back}$, $CL_{int,bile}$, and $CL_{int,met}$.

If the drug is not substrate of hepatic drug transporters and rapidly penetrates the sinusoidal membrane, i.e., $CL_{int,up} = CL_{int,back}$, thus, $CL_{int,all}$ becomes sum of $CL_{int,bile}$ and $CL_{int,met}$. In this case, the exact intrinsic clearance of drugs is determined by metabolic rates and biliary excretion rates, which is the conventional clearance concept. If drug is extensively metabolized in the liver and excretion via bile may be negligible, traditional in vitro method of hepatic clearance prediction using human hepatic microsomes can be effective.

In general, hepatic uptake of OATP substrates is mainly controlled by uptake transporter OATPs and hepatic uptake is rate-limited process in their hepatic clearances. The widely investigated drugs are statins. For example, atorvastatin uptake into hepatocytes is mediated by the OATPs and subsequently metabolized by CYP3A4. A clinical study (Maeda et al. 2011) showed that coadministration of rifampicin (an inhibitor of OATP1B1) induced 12-fold and 4.6-fold in AUC of atorvastatin and pravastatin, respectively. But, intravenous coadministration of itraconazole (a potent inhibitor of CYP3A4) did not affect AUC of atorvastatin, although coadministration of itraconazole significantly increased the AUC of midazolam (a substrate of CYP3A4) and significantly decreased the plasma concentration of 1-hydroxyatorvastatin, a major metabolite of atorvastatin produced by CYP3A4. Cyclosporine A also markedly raised the plasma concentrations of some statins (Niemi et al. 2011). The largest extent of increase (20-fold) in *AUC* by cyclosporine A was lovastatin, followed by atorvastatin, pravastatin, rosuvastatin, simvastatin, pitavastatin, cerivastatin, and fluvastatin. The increased *AUC* for simvastatin, lovastatin, atorvastatin, and cerivastatin by cyclosporine A may be partly explained using inhibition of CYP3A4, but not the other statins rosuvastatin, pravastatin, and pitavastatin which are little metabolized by CYP3A4. In vitro-in vivo extrapolation of overall intrinsic hepatic clearances for pravastatin, pitavastatin, atorvastatin, and fluvastatin using uptake clearance ($CL_{int,up}$) or metabolic clearance ($CL_{int,met}$) both in rats and human were investigated (Watanabe et al. 2010a). The hepatic uptake clearances and metabolic clearances were estimated, and the parameters are scaled up to in vivo data and compared with overall intrinsic clearance. The estimated uptake clearance of the statins in humans (1.44 mL/min/g liver for pravastatin, 30.6 mL/min/g liver for pitavastatin, 12.7 mL/min/g liver for atorvastatin, and 62.9 mL/min/g liver for fluvastatin) from the isolated hepatocytes was within the range of overall in vivo intrinsic hepatic clearance (0.84~1.2 ml/min/g

liver for pravastatin, 14~35 mL/min/g liver for pitavastatin, 11~19 mL/min/g liver for atorvastatin, and 123~185 mL/min/g liver for fluvastatin), whereas the scaled metabolic clearance of pitavastatin (0.248 mL/min/g liver), atorvastatin (2.98 mL/min/g liver), and fluvastatin (5.57 mL/min/g liver) from liver microsomes was greatly lower than overall intrinsic clearances of the statins. Similar phenomenon was observed in rats. The results clearly demonstrated that in vivo hepatic clearances of these statins were well correlated with in vitro uptake intrinsic clearances in isolated hepatocytes not in vitro metabolic intrinsic clearances from liver microsomes. These results infer that the overall hepatic clearance of the four statins is mainly dominated by the hepatic uptake process.

Hepatocytes often coexpress many uptake transporters such as OATP1B1, OATP1B3, OATP2B1, organic anion transporter 2 (OAT2), organic cation transporter 1 (OCT1), and sodium taurocholate cotransporting polypeptide (NTCP). Some drugs often exhibit overlaps of transporter specificity; thus, drug uptake by hepatocytes is the sum of all transporter-specific contributions. Therefore, the contribution of each isoform to hepatic uptake should be investigated when predicting drug-drug interactions. The methods widely used for assessing their contributions include relative activity factors (RAF) and relative transporter expression factors (REF). HEK293 cell lines expressing OATP1B1 or OATP1B3 are often served as in vitro model. E3S and CCK-8 are usually used as OATB1B1 and OATP1B3 probes, respectively.

For RAF method, value of RAF_i for the targeted transporter is defined as ratio of the transporter activities of transporter-specific probes in hepatocytes to that in HEK293 cell lines expressing transporters (Eq. 3.2). The uptake clearances of the tested drugs mediated by the *i*th transporter are calculated by multiplying the uptake clearance of the test compounds in transporter-expressing cells ($CL_{\mathrm{int},C,\,\mathrm{test}}$) by RAF_i (Eq. 3.3). Total uptake clearance of the tested drug and contribution of the *i*th transporter are estimated using Eqs. 3.4 and 3.5, respectively.

$$\mathrm{RAF}_i = CL_{\mathrm{int},H,i}/CL_{\mathrm{int},C,i}. \tag{3.2}$$

$$CL_{\mathrm{int},H,\mathrm{test},i} = CL_{\mathrm{int},C,\mathrm{test}} \times \mathrm{RAF}_i. \tag{3.3}$$

$$CL_{\mathrm{int,tot}} = \sum CL_{\mathrm{int},H,\mathrm{test},i} \tag{3.4}$$

$$\mathrm{Contribution}(\%) = CL_{\mathrm{int},H,\mathrm{test},i}/CL_{\mathrm{int,tot}} \times 100\% \tag{3.5}$$

where $CL_{\mathrm{int},H,i}$ and $CL_{\mathrm{int},C,i}$ are uptake clearance for transporter-specific probe i in hepatocytes and HEK293 cells expressing transporter, respectively. $CL_{\mathrm{int},H,\mathrm{test},i}$ is uptake clearance of the tested compound mediated by the *i*th transporter in hepatocytes. $CL_{\mathrm{int},H,\mathrm{test}}$ and $CL_{\mathrm{int},C,\mathrm{test}}$ are uptake clearance for the tested compound in hepatocytes and HEK293 cells expressing transporter i, respectively.

For REF method, value of $\mathrm{REF_i}$ for the *i*th transporter is calculated using absolute transporter protein expression data of the *i*th transporter in hepatocytes ($PEX_{H,i}$) and HEK293 cells expressing transporter ($PEX_{C,i}$).

$$\mathrm{REF}_i = PEX_{H,i}/PEX_{C,i} \tag{3.6}$$

And $CL_{\mathrm{int},H,\mathrm{test},i}$ in hepatocytes is also estimated using Eq. 3.7.

$$CL_{\mathrm{int},H,\mathrm{test},i} = CL_{\mathrm{int},C,\mathrm{test}} \times \mathrm{REF}_i \tag{3.7}$$

where the protein expression data of the transporter may be measured using Western blot (Hirano et al. 2004) or liquid chromatography-tandem mass spectrometry (Kunze et al. 2014; Karlgren et al. 2012; Vildhede et al. 2014).

Relative contributions of OATP1B1- and OATP1B3-mediated transport to hepatic clearance of olmesartan, a substrate of OATP1B1 and OATP1B3, were investigated using the RAF method (Yamada et al. 2007). The predicted sum (1.58 μL/min/10^6cells) of $CL_{\mathrm{int},OATP1B1}$ and $CL_{\mathrm{int},OATP1B3}$ was comparable to values of observed CL_{int} (2.16 μL/min/10^6 cells). The estimated relative contribution (37.34%) of OATP1B1 to the uptake of olmesartan into human hepatocytes was almost similar to that of OATP1B3 (36.11%) in spite of a large variation among the three batches of human hepatocytes. Kunze et al. (2014) compared relative contribution of OATP1B1- and OATP1B3-mediated uptake of eight statins (atorvastatin, cerivastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin) into hepatocytes using RAF method and REF method, where protein expression was detected by LC-MS/MS. The results showed that predictions using RAF and REF methods were highly similar, the ratios of two predictions were 0.75~0.88, indicating a direct transporter expression-activity relationship. For all statins, OATP1B1-mediated uptake into hepatocytes was significantly higher than uptake by OATP1B3, which is consistent with higher expression of OATP1B1 protein. Moreover, the hepatic uptake clearances of atorvastatin, pravastatin, rosuvastatin, and simvastatin were well predicted within twofold error, demonstrating that OATP1B1 and OATP1B3 were major contributors. But, the hepatic uptake clearance of other statins was greatly underestimated, demonstrating roles of other hepatic uptake transporters.

However, another report showed that predicated sum clearances of OATP1B1- and OATP1B3-mediated transport to pitavastatin and $E_2$17βG in human hepatocytes using RAF were almost identical to the observed uptake clearances, but predicted sum clearance of the OATP1B1- and OATP1B3-mediated uptake of pitavastatin and $E_2$17βG using REF method were five to ten times higher than their observed clearance in hepatocytes (Hirano et al. 2004). Moreover, total contribution of OATB1B1- and OATP1B3-mediated uptake to hepatic uptake of pitavastatin (86.2%) by Hirano et al. (2004) was higher than that (34.9%) by Kunze et al. (2014). These discrepancies may partly result from large differences in the expression and function of the hepatic OATPs (Vildhede et al. 2014; Yamashiro et al. 2006). For example, Vildhede et al. (2014) reported that varieties of NTCP, OATP1B1, OATP1B3, and OATP2B1 protein expression among 12 sample donors were 7.5, 3.7, 32.0, and 7.5, respectively. Based on the substantial variation in transporter protein abundance among the investigated liver samples (12 donors), total contribution of transporter-mediated uptake was

predicted to be 90% of overall atorvastatin uptake, but the predicted transporter-specific uptake clearances showed high inter-individual variability, with relative contributions of 26–89%, 1.8–60%, 3.2–30%, and 1.5–10% of the total active uptake for OATP1B1, OATP1B3, OATP2B1, and NTCP, respectively. Similarly, the summed contributions of OATP1B1 and OATP1B3 to hepatic uptake of valsartan in human hepatocytes showed great difference, ranging from 19% to 95% in the three batches of human hepatocytes (Yamashiro et al. 2006), of which, differences in contributions of OATP1B1 and OAT1B3 were from 13.4% to 48.8 %, from 5.5% to 46.5% , respectively.

Although RAF method may give good results for estimating contribution of transporter to uptake clearance, it should be minded to that the selectivity of probe substrates is critical. Uptakes of E3S are often used to assess function activities of OATP1B1, but E3S is also substrates of OATP2B1. OATP2B1 is detected at a comparable level in human liver (Kimoto et al. 2012; Vildhede et al. 2014), indicating that E3S hepatic uptakes may be mediated not only by OATP1B1, but also by OATP2B1, which seems to partly explain the finding that the estimated contributions of OATP1B1 to hepatic uptake clearance of the tested statins using RAF method were larger than those by REF method (Vildhede et al. 2014).

An isoform-selective inhibitor was used to investigate contribution of OATP1B3 and OATP1B1 to hepatic uptake clearance of telmisartan (Ishiguro et al. 2006). In HEK293 cells expressing transporter, E3S inhibited OATP1B1-mediated $E_2$17βG uptake with an IC_{50} value of 0.8 μM, but not inhibited OATP1B3-mediated CCK-8 uptake up to 30 μM, inferring that 30 μM E3S can selectively inhibit the OATP1B1-mediated uptake. In consistence with this, the uptake of telmisartan into OATP1B3-expressing cells and cryopreserved human hepatocytes was not inhibited by 30 μM E_3S, but 30 μM E3S inhibited more than half of $E_2$17βG uptake in all batches of human hepatocytes (Ishiguro et al. 2006). This approach is very easy to take, but it is important to identify and select isoform-selective inhibitor. Therefore, whether the isoform-selective inhibitor inhibits the uptake of substrates in targeted transporter-expressing cells should be checked beforehand. It should be pointed out that each method has some advantages and disadvantages compared with other methods; thus the relative contribution of each isoform must be confirmed by multiple approaches.

The clinical significance of OATP1B1 in disposition of drugs has been emphasized since molecular mechanism of OATP-mediated drug-drug interaction (DDI) for cerivastatin was identified. The use of cerivastatin was linked to rhabdomyolysis and was responsible for 52 fatalities, and in some cases, gemfibrozil was coadministered with cerivastatin (Furberg and Pitt 2001). Clinical studies demonstrated that coadministration of gemfibrozil (Backman et al. 2002) and cyclosporine A (Mück et al. 1999) increased in cerivastatin plasma concentrations by threefold and fivefold, respectively. Cerivastatin is metabolized by both CYP2C8 and CYP3A4. It was once believed that DDI for cerivastatin was due to inhibition of these CYP450 enzymes. However, in vitro data showed demonstrated that cyclosporine A significantly inhibited OATP-mediated hepatic uptake of cerivastatin into human hepatocytes or OATP1B1-expressing MDCKII cell lines, whose K_i values were less than 1 μM (Shitara et al. 2003). Although cyclosporine A also inhibited cerivastatin metabolism in vitro study,

but its IC_{50} value was more than 30 μM (Shitara et al. 2003), inferring that DDI between cerivastatin and cyclosporine A is mainly due to the inhibition of OATP1B1-mediated hepatic uptake. Coadministration of erythromycin (a potent mechanism-based inhibitor of CYP3A4) did not affect the plasma concentrations of cerivastatin (Mück et al. 1998), supporting above deduction. Similarly, coadministration of cyclosporine A induced about 20-fold increase in *AUC* of pravastatin (not a substrate of CYP3A4) (Regazzi et al. 1993). Gemfibrozil and its metabolite gemfibrozil-1-O-β-glucuronide also significantly inhibited the OATP1B1-mediated uptake of cerivastatin (Shitara et al. 2004), with IC_{50} values of 72 and 24 μM, respectively. The two compounds also inhibited the CYP2C8-mediated metabolism of cerivastatin with IC_{50} values of 28 and 4 μM, respectively, but minimally inhibited the CYP3A4-mediated metabolism of cerivastatin. These results indicate that inhibition of both CYP2C8 and OATP1B1 by gemfibrozil-1-O-β-glucuronide is a major cause of the DDI. Accumulating examples of DDIs mediated by hepatic OATP transporters have been reported a 2- to 20-fold increase in *AUC* of several OATP substrates with inhibitory drugs such as cyclosporine A, rifampicin, and gemfibrozil (Brennan et al. 2013; Chen et al. 2013; Hu 2013; Maeda et al. 2011; Shitara 2011; Shitara and Sugiyama 2006) (Fig. 3.4), leading to the some regulatory agencies to add OATP1B1 and OATP1B3 as important target sites of DDIs in their guidance.

Rifampicin is another inhibitor of OATP1B1 and OATP1B3, although it also is an inducer of metabolic enzymes and transporters. Thus, type of DDI between rifampicin and substrate drugs is often dependent-time course. For example, Lau et al. (2007) reported that single intravenous dose of rifampicin significantly increased *AUC* of atorvastatin acid by 6.8-folds. On the contrast, 5-day pre-treatment with oral dose rifampicin reduced *AUC* of atorvastatin acid by 80% (Backman et al. 2005), which may be attributed to CYP3A4 induction.

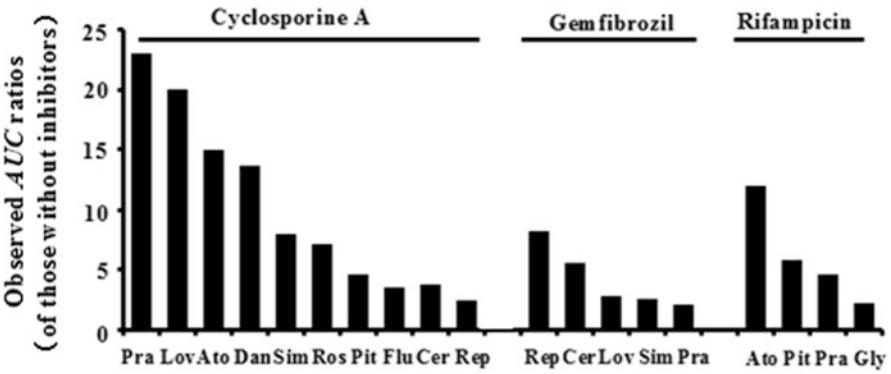

Fig. 3.4 The increased plasma *AUC* of several OATP substrate drugs by coadministration of cyclosporine A, gemfibrozil, and rifampicin in human. Symbol: Pra, pravastatin; Lov, lovastatin; Ato, atorvastatin; Dan, danoprevir; Sim, simvastatin; Ros, rosuvastatin; Pit, pitavastatin; Flu, fluvastatin; Cer, cerivastatin; Rep, repaglinide; Gly, glyburide. Graph data were cited from literatures. (Brennan et al. 2013; Chen et al. 2013; Hu 2013; Maeda et al. 2011; Shitara 2011; Shitara and Sugiyama 2006)

Other drugs such as HIV protease inhibitors lopinavir and ritonavir are also inhibitors of OATP1B1. Coadministration of lopinavir/ritonavir (Kiser et al. 2008) or atazanavir/ritonavir (Busti et al. 2008) induced increases in *AUC* and C_{max} of rosuvastatin by 2.1- and 4.7-6.0 folds, respectively, which may become a reason leading to severe rhabdomyolysis of rosuvastatin with HIV protease inhibitors (de Kanter et al. 2011; Moreno et al. 2011). Moreover, coadministration of lopinavir/ritonavir significantly increased steady-state *AUC* and C_{max} of bosentan by 5.2- and 6.1-folds, respectively, leading to an increase in adverse events (Dingemanse et al. 2010). Clarithromycin is OATP1B1 inhibitor and potent mechanism-based inhibitor of CYP3A4. Coadministration of clarithromycin (Jacobson 2004) also significantly increased the *AUC* of atorvastatin (4.5-fold increase), simvastatin (10-fold), simvastatin acid (12-fold), and pravastatin (2.1-fold), respectively. Atorvastatin and simvastatin but not pravastatin may be metabolized by CYP3A4, indicating that DDIs between these statins and clarithromycin are at least partly attributed to OTAP1B1 inhibition.

OATP1B1 also mediates transport of certain endogenous compounds such as bilirubin, bile acids, steroid and thyroid hormones, and their conjugates, inferring that abnormity of the transporter may be implicated in pathophysiological process. Rotor syndrome is a rare, benign hereditary conjugated hyperbilirubinemia. van de Steeg et al. (2012) investigated Rotor syndrome index subjects from eight different families. Their results revealed that the homozygous inactivation of two adjacent genes of *SLCO1B1* and *SLCO1B3* is necessary to cause a Rotor syndrome phenotype. *Slco1a/1b*$^{-/-}$ mice exhibited a phenotype similar to Rotor syndrome, which was rescued by transgenic expression of human OATP1B1 or OATP1B3. Similarly, Kagawa et al. (2015) reported that all six Japanese patients with Rotor syndrome had a single causative haplotype which consists of the c.1738C>T(p.R580X) nonsense mutation in *SLCO1B1* and the long-interspersed elements insertion in intron 5 of *SLCO1B3*. Moreover, hyperbilirubinemia induced by some drugs such as simvastatin and atorvastatin (Bergmann et al. 2012; Lopez and Tayek 2016) may be partly explained using direct inhibition of OATP1B1/OATP1B3, although contributions of other mechanisms such as MRP2, MRP3, OATP1B1, and OATP1B3 (Chang et al. 2013; Keppler 2014) are not excluded.

OATP1B1 accepts thyroid hormones as exogenous substrates. It was reported (Meyer zu Schwabedissen et al. 2011) that that lack of Oatp1b2 significantly reduced expression of Cyp7a1 and hepatic glucose transporter 2(Glut2), resulting in elevated cholesterol levels and delayed clearance of glucose after oral glucose challenge. These alterations may be partly attributed to reduction in thyroid hormone hepatocellular entry via Oatp1b2. Data from a cohort of archived human livers also demonstrated high association of OATP1 expression with target genes of thyroid hormone receptor, especially for GLUT2. Mouse experiment showed that Oatp1b2 deficiency increased the serum concentrations of unconjugated bile acids by 3–45-folds of wild-type mice. Pharmacokinetic analysis showed that clearance of cholate following intravenous administration of cholate was significantly decreased in *Slco1b2*$^{-/-}$ mice compared with wild-type mice (Csanaky et al. 2011), inferring roles of Oatp1b2 in hepatic uptake of unconjugated bile acids.

Some diseases may affect OTAP1B1 expression and function. Clinical study (Sticova et al. 2015) showed that expression of the OATP1B proteins was decreased in advanced liver diseases and inversely correlated with serum bilirubin levels. The reduction was more pronounced in advanced primary biliary diseases. Animal experiments demonstrated that alteration of Oatps by disease also affected disposition of drug and its toxicity. We once reported that diabetes induced function and expression of hepatic Oatp1b2 and hepatic Cyp3a in rats, leading to enhancement of atorvastatin hepatotoxicity (Shu et al. 2016a). Phenobarbital also aggravated atorvastatin-induced hepatotoxicity via inducting hepatic Cyp3a and Oatp1b2. Diabetic rats also exhibited low plasma exposure of atorvastatin and simvastatin compared with normal rats (Shu et al. 2016b; Xu et al. 2014) due to induction of hepatic Cyp3a and Oatp1b2. On the contrast, nonalcoholic steatohepatitis rats by a methionine- and choline-deficient diet showed higher plasma exposure of simvastatin acid compared with control rats. Low hepatic-to-plasma ratio of simvastatin acid was observed, which was consistent with decreased expression of Oatp1b2 and Oatp1a1 (Clarke et al. 2014).

3.2.2.4 OATP1B1 Polymorphisms and Drug Disposition

The more than 40 nonsynonymous variants of *SLCO1B1* have been identified (Niemi et al. 2011), and 14 SNPs in 15 haplotypes were systematically investigated (Tirona et al. 2001). In vitro function analysis using three probes ($E_2$17βG, E3S, and rifampicin) demonstrated that several variants (such as ∗2 *c.217T>C*, ∗3 *c.245T>C and c.467A>G*, ∗5 *c.521T>C*, ∗6 *c.1058T>C*, ∗7 *c.1294A>G*, ∗9 *c.1463G>C*, ∗12 *c.217T>C* and *c.467A>G*, ∗13 *c.245T>G*, and *c.467A>G*) decreased transport activity of OATP1B1 (Tirona et al. 2001), some of which (such as ∗2,∗3,∗5,∗6, and ∗9) also affected cell membrane surface of OATP1B1 (Gong and Kim 2013). One of the relatively common *SLCO1B1* variants is *c.521T>C* (p.Val174Ala) in exon 5, resulting in the decrease in the maximum transport velocity with little affecting E3S affinity (Tirona et al. 2001; Gong and Kim 2013). The *c.521T>C* variant also shows lower transport activities for other substrates such as rifampin, pravastatin, atorvastatin, rosuvastatin, cerivastatin, atrasentan, and ezetimibe glucuronide (Gong and Kim 2013). Another common *SLCO1B1* variant associated with altered transport activity of OATP1B1 is *c.388A>G*(p.Asn130Asp) in exon 4. Reports about effect of *c.388A>G* on OATP1B1 function appear to be controversial. The *c.388A>G* was reported to be associated with increased OATP1B1 transport activity in studies using BSP and E3S as probes, whereas no change or reduced transport activity has been seen in other studies with different substrates, which may be explained in part by a substrate-specific effect of the variant and/or the use of different expression systems or experimental conditions (Gong and Kim 2013; Kalliokoski and Niemi 2009).

It is noteworthy that the two common SNPs (*c.388A > G* and *c.521T > C*) form four distinct haplotypes, known as ∗1A (*c.388A-c.521T*, reference haplotype), ∗1B (*c.388G-c.521T*), ∗5(*c.388A-c.521C*), and∗15(*c.388G-c.521C*). The *SLCO1B1∗15*

haplotype can be further subclassified on the basis of two promoter variants, *g.−11187G > A* and g.*−10499A > C*, forming the ∗15(GAGC), ∗16(GCGC), and ∗17 (AAGC) haplotypes. The haplotype frequencies for the *SLCO1B1∗1A*, *∗1B*, *∗5*, *∗15*, *∗16*, and *∗17* were reported to be 52, 27, 2.7, 2.4, 7.9, and 6.9%, respectively, in a population of 468 healthy Finnish Caucasian subjects (Pasanen et al. 2006a). Interestingly, *SLCO1B1∗15* haplotype has been consistently associated with decreased transport activity using $E_2$17βG, pravastatin, atorvastatin, rosuvastatin, and cerivastatin uptake, inferring that the effect of the *c.521T>C* SNP appears to dominate over that of the *c.388A>G* SNP. Characteristics of several OATP1B1 haplotypes were list in Table 3.3.

Frequencies of *SLCO1B1* variants show large race differences. For example, the *c.388A > G* variant is quite common in all populations, with an allele frequency ranging from ~40% in Europeans to ~80% in sub-Saharan Africans and East Asians, whereas the *c.521T > C* (p.Val174Ala) SNP, relatively common in Europeans and Asians (allele frequency ~10–20%), is less frequent in sub-Saharan Africans (~2%) and Oceania (0%) (Pasanen et al. 2008). Similarly, the low-activity haplotypes *SLCO1B1∗5* and *∗15* have a combined frequency of approximately 15–20% in Europeans, 10–15% in Asians, and 2% in sub-Saharan Africans. The *SLCO1B1∗1B* haplotype has a frequency of approximately 26% in Europeans, 39% in South/Central Asians, 63% in East Asians, and as high as 77% in sub-Saharan Africans (Niemi et al. 2011).

Nishizato et al. (2003) first reported the in vivo pharmacokinetic effect of *SLCO1B1* variants on pravastatin. The results showed that nonrenal clearance of pravastatin in persons with the *SLCO1B1∗1B/∗15* genotype was only 50% of ∗1B/∗1B genotype

Table 3.3 Several *SLCO1B1* haplotypes and their function. Data were cited from previous reports. (Gong and Kim 2013; Kalliokoski and Niemi 2009; König et al. 2006; Pasanen et al. 2006a; Tirona et al. 2001; Zaïr et al. 2008)

Allele	Nucleotide change	Function
∗1B	*c.388A>G*	Increased
∗2	*c.217T>C*	Decreased
∗3	*c.245T>C+c.467A>G*	Decreased
∗4	*c.463C>A*	Unchanged
∗5	*c.521T>C*	Unchanged
∗6	*c.1058T>C*	Decreased
∗7	*c.1294A>G*	Decrease
∗9	*c.1463G>C*	Decreased
∗11	*c.2000A>G*	Unchanged
∗12	*c.217T>C+c.1964A>G*	Decreased
∗13	*c.217T>C+c.467A>G+c.2000A>G*	Decreased
∗14	*c.388A>G+c.463C>A*	Unchanged
∗15	*c.388A<G+c.521T>C*	Decreased
∗16	*c.388A<G+c.521T>C+ g.−10499G>A*	Undetected
∗17	*c.388A<G+c.521T>C+g.−11187G>A*	Decreased
∗18	*c.388A>G+411G>A+464C>A+578T>G*	Impaired surface expression

carriers, which was consistent with a reduced hepatic uptake in the ∗15 haplotype. Similarly, Ho et al. (2007) reported that a 45% higher AUC for pravastatin in subjects heterozygous for *SLCO1B1∗15* and 92% higher in subjects homozygous for *SLCO1B1∗15* compared with subjects homozygous for *SLCO1B1∗1A*. Another report showed that *AUC* of pravastatin in *SLCO1B1∗1B/∗1B* carriers was only 65% of that in *∗1A/∗1A* carriers and that carriers of *∗1B/∗15* exhibited a 45% lower *AUC* than *∗1A/∗15* carriers (Maeda et al. 2006). Moreover, Mwinyi et al. (2004) compared pharmacokinetics of pravastatin in following a single oral dose of 40 mg pravastatin to *SLCO1B1* ∗1A/∗1A, ∗1A/∗1B, or *∗1B/∗1B+∗1A/∗5* individuals. The results demonstrated highly significant differences across all three study groups and between subjects carrying the *∗1B* and *∗5* haplotypes. The ranks of AUC^{0-6h} values for pravastatin were *∗1A/∗1B* individuals (74.8±68.6 μg.h/L)<*1A/∗1A* individuals (114.5 ± 68.6 μg.h/L) <*∗1B/∗1B+∗1A/∗5* individuals (163.0 ± 64.6 μg.h/L). Strikingly, values of AUC^{0-6h} from the *SLCO1B1∗*1B group were more than 60% lower than those derived from carriers of the wild-type *SLCO1B1∗*1A haplotype, although no statistical significance was obtained (Mwinyi et al. 2004). These results indicate greater hepatic uptake in subjects with ∗1B genotype. Effects of *SLCO1B1* polymorphisms on pharmacokinetics of other statin have been also demonstrated. *AUCs* of simvastatin acid in participants with the *SLCO1B1 c.521CC* genotype (Pasanen et al. 2006b) were reported to be 120 and 221% higher than those in subjects with the *c.521TC* and *c.521TT* (reference) genotypes, respectively. AUC^{0-48h} values of atorvastatin in subjects with the *SLCO1B1 c.521CC* genotype were 61% and 144% greater than those in subjects with the *c.521TT* and *c.521TC* genotype, respectively. Similarly, *AUC* of rosuvastatin in subjects with the *c.521CC* genotype was also 65% higher than in those with the *c.521TT* genotype (Pasanen et al. 2007).

The altered pharmacokinetics caused by *SLCO1B1* variants can translate to altered therapeutic and adverse drug response. Targeted tissue of statins is the liver, indicating that *SLCO1B1* SNPs may attenuate cholesterol-lowering effects of statins due to decreases in hepatic uptake. A retrospective study showed that patients carrying *c.521TC* genotype had a 22.3% attenuation in the total cholesterol-lowering action of pravastatin, atorvastatin, and simvastatin compared with *c.521TT* carriers (Tachibana-Iimori et al. 2004). In 45 Chinese coronary heart disease patients, it was reported that patients carrying *c.521TC* genotype showed significantly lower total cholesterol reduction relative to 521TT genotype (14.5% versus 22.4%) following treatment with pravastatin 20 mg/day for 30 days (Zhang et al. 2007). Conversely, subjects carrying *c.388GG* genotype exhibited significantly higher low-density lipoprotein cholesterol (LDL-C) reduction relative to *c.388AA+c.388AG* carriers (41 versus 37%) following oral atorvastatin (10 mg/day for 4 weeks) (Rodrigues et al. 2011). A multivariate logistic regression analysis also confirmed that *c.388GG* genotype was associated with higher LDL-C reduction following statin treatment, inferring higher transport function for OATP1B1 in subjects carrying *SLCO1B1∗1B* variant. Similarly, in 129 Chilean hypercholesterolemic patients, it was reported carriers of *c.388GG* genotype displayed greater mean values of low-density lipoprotein cholesterol (HDL-C) than *c.388AA* genotype (change: 11.6% for *c.388AA*, 20.0% for *c.388AG* and 22.5% for *c.388GG*, respectively) following 10 atorvastatin

mg/day for 4 weeks (Prado et al. 2015). It is noteworthy that these effects are relatively modest and may not translate to dramatic alterations in patient outcomes.

Several studies have linked simvastatin-induced myopathy with the *c.521T > C* SNP (Link et al. 2008; Brunham et al. 2011; Voora et al. 2009). A genome-wide association study demonstrated that *c.521CC*, *c.521CT*, and *c.521TT* genotype had an 18%, 3%, and 0.6% cumulative risk, respectively, with myopathy during the first year following taking 80 mg of simvastatin. Overall, more than 60% of these myopathy cases could be attributed to *c.521C* variant (Link et al. 2008). Another genome-wide association study also showed that occurrence of a composite adverse event induced by statins was associated with gene-dose effect of *SLCO1B1∗5*. The proportions with the composite adverse event for non-carriers (n = 325), carriers of one allele (n = 115), and carriers of two alleles (n = 8) were reported to be 0.19, 0.27, and 0.50, respectively (Voora et al. 2009). A follow-up study demonstrated that patients carrying *c.521C* variant had a threefold increased risk for myopathy following simvastatin treatment. But, the association between the *c.521C* variant and myopathy was not found in patients taking atorvastatin (Brunham et al. 2011). A meta-analysis on 9 studies with 1360 cases and 3082 controls (Hou et al. 2015a) demonstrated that cases of statin-related myopathy were significantly associated with the *c.521TC* allele (*TC+CC* versus *TT*, OR = 2.09; and *C* versus *T*, OR = 2.10), especially when statin-related myopathy was defined as an elevation of creatine kinase >10 times the upper limit of normal or rhabdomyolysis (*TC+CC* versus *TT*, OR = 3.83; and *C* versus *T*, OR = 2.94). When stratified by statin type, the association was significant in individuals receiving simvastatin (*TC+CC* versus *TT*, OR = 3.09; *C* versus *T*, OR = 3.00), but not in those receiving atorvastatin, inferring that *SLCO1B1 T521C* polymorphism is associated with an increased risk of statin-related myopathy, especially in individuals receiving simvastatin. Limiting analysis showed that in patients receiving simvastatin not atorvastatin, statistically significant associations between *c.521T>C* and risk of both myopathy and severe myopathy (Carr et al. 2013). All these results indicate that statin-induced myopathy with *c.521T>C* variants is simvastatin specific, although a report showed significant association between *SLCO1B1 521C* mutant allele mutation and risk of myotoxicity in Chinese individuals (*CC*+TC versus *TT*,OR = 3.67) that received rosuvastatin but not atorvastatin nor simvastatin (Liu et al. 2017).

Effects of *SLCO1B1* SNPs on pharmacokinetics and pharmacodynamics/toxicity of other substrate drugs have been investigated. It was reported that participants carrying *c.521CC* genotype (Kalliokoski et al. 2008) showed 59% or 72% greater the repaglinide *AUC* following oral dose (0.5 mg) than participants carrying *c.521TC* or *c.521TT* genotype. In accordance, the participants carrying *c.521CC* genotype showed a tendency toward a greater blood glucose-lowering effect of repaglinide than participants carrying *c.521TC* or *c.521TT* genotype. The mean maximum increases in blood glucose (after meals) were reported to 0.8 mmol/L in *c.521CC* participants, 1.3 mmol/L in *c.521TC* participants, and 1.5 mmol/L in *c.521TT* participants following 0.5 mg of repaglinide, respectively. Similarly, patients carrying at least one *SLCO1B1∗15* haplotype were reported to have a significantly increased risk for cholestatic/mixed injury by rifampin (Li et al.

2012). In vitro study showed that bile acid uptake by *SLCO1B1∗15* haplotype was markedly reduced compared to the other haplotypes. Moreover, *SLCO1B1∗15* haplotype showed stronger inhibition on bile acid by rifampin compared to that in the other haplotypes. Similar report (Chen et al. 2015a) showed that patients carrying at least one *SLCO1B1∗15* haplotype had a significantly higher risk of antituberculosis drug-induced hepatotoxicity in comparison with those carrying *SLCO1B1∗1A* or *SLCO1B1∗1B* haplotypes.

3.2.3 OATP1A2

OATP1A2 (also known as human OATP-A or OATP1) is a 670-amino acid glycoprotein coded by *SLCO1A2*. Human OATP1A2 has five rodent orthologues, Oatp1a1, Oatp1a3 (in rats only), Oatp1a4, Oatp1a5, and Oatp1a6, whose amino acid sequence identities with human OATP1A2 (Hagenbuch and Meier 2003; Tamai 2012) are 66%, 66%, 73%, 72%, and 67 %, respectively.

OATP1A2 has broad substrate specificity including endogenous amphipathic substrates and therapeutic drugs. Endogenous substrates of OATP1A2 include bile acids (such as cholate, tauroursodeoxycholate, aurochenodeoxycholate, taurocholate, and glycocholate), steroid hormones and their conjugates (such as E3S, $E_2$17βG, and DHEAS), thyroid hormones (thyroxin and triiodothyronine), prostaglandins (PGE2), neuropeptides (substance P and vasoactive intestinal peptide), and all-trans-retinol (Kalliokoski and Niemi 2009; Chan et al. 2015; Gao et al. 2015). Clinically important drug substrates include antibiotics (such as ciprofloxacin, enoxacin, erythromycin, gatifloxacin, lomefloxacin, levofloxacin, norfloxacin, and tebipenem pivoxil), anticancer agents (docetaxel, doxorubicin, hydroxyurea, imatinib, methotrexate, and paclitaxel), antihistamine drug (fexofenadine), β-blockers (atenolol, acebutolol, celiprolol, labetalol, nadolol, sotalol, and talinolol), HIV protease inhibitors (darunavir, lopinavir, and saquinavir), statins (pitavastatin, pravastatin, and rosuvastatin), and other drugs (atrasentan, BSP, N-methylquinine, N-methylquinidine, ouabain, and rocuronium) (Kalliokoski and Niemi 2009; Hartkoorn et al. 2010; König et al. 2006; Shitara et al. 2013a; Xu et al. 2013; Xu et al. 2016, Zhou et al. 2015). Most of OATP1A2 substrates are often substrates of other transporters such as OATP1B1, OATP2B1, and P-GP. A series of compounds have been identified as inhibitors of OATP1A2 such as atorvastatin, dexamethasone, hesperidin, naloxone, naringin, rifampicin, saquinavir, verapamil, chloroquine, hydroxychloroquine, atorvastatin, dexamethasone, naloxone, rifampicin, saquinavir, verapamil, and flavonoids. Flavonoids in vegetables, fruits, and plants are inhibitors of OATP1A2, which may lead to food-drug interaction via inhibiting intestinal OATP1A2 function (Bailey 2010; Bailey et al. 2007) when they are coadministrated to human.

The tissue distribution of OATP1A2 appears ubiquitous. The highest mRNA expression is in the brain, followed by the kidney, liver, lung, and testes. OATP1A2 protein is expressed in the luminal membrane of the brain capillary endothelial cells, apical membranes of cholangiocytes in the liver, apical membrane of the distal

nephrons in the kidney, and apical membrane of enterocytes in the duodenum (Glaeser et al. 2007; Hartkoorn et al. 2010), in red blood cells (Chan et al. 2015), and in the retina (Gao et al. 2015). Tissue distribution of OATP1A2 implies to be linked with drug disposition including brain distribution, reabsorption in the bile, reabsorption or secretion in the kidney, and intestinal absorption of substrate drugs.

In intestine, OATP1A2 protein is localized to the brush-border membrane of enterocytes in the duodenum, where it mediates the absorption of therapeutic drugs. Several studies have demonstrated that ingestion of some fruit juices may result in remarkable food-drug interaction via inhibiting intestinal OATP1A2 function, confirming roles of OATP1A2 in intestinal absorption. For example, OATP1A2 was identified to be the key intestinal fexofenadine uptake transporter (Glaeser et al. 2007). It was found that ingestions of grapefruit juice at 0 and at 2 h before fexofenadine reduced fexofenadine AUC^{0-8h} by 52% and 38%, respectively. But no effect was observed at 4 h following grapefruit juice (Glaeser et al. 2007). Similarly, grapefruit grape juice, orange juice, or apple juice also significantly decreased AUC and C_{max} of OATP substrate drugs including atenolol (Lilja et al. 2005), atenolol (Jeon et al. 2012), celiprolol (Lilja et al. 2003; Lilja et al. 2004), and fexofenadine (Akamine et al. 2014; Akamine et al. 2015), via inhibiting intestinal OATP1A2 or OATP1B2 activity. In addition, green tea markedly decreased the C_{max} and AUC^{0-48h} values of nadolol by about 85% (Misaka et al. 2014).

OATP1A2 is expressed at the apical membrane of the distal nephron, where it could be responsible for either the reabsorption of xenobiotics from or their secretion into urine. OATP1A2 mediates the active tubular reabsorption of methotrexate, indicating that OATP1A2 may be implicated in methotrexate-induced toxicities (Zhou et al. 2015; Badagnani et al. 2006). OATP1A2 is also expressed in the cholangiocytes of the liver and in the brain capillary endothelium, suggesting that OATP1A2 may be involved in the reabsorption of xenobiotics excreted into the bile and transport of many drugs across the blood-brain barrier (BBB) (Lee et al. 2005; Gao et al. 2015).

OATP1A2 is also expressed in plasma membrane of red blood cells (Hubeny et al. 2016). Some important antimalarial drugs including quinine and chloroquine, acting against blood schizonts by interfering with hemoglobin metabolism, are also substrate of OATP1A2. Moreover, quinine, chloroquine, and hydroxychloroquine are potent inhibitors of OATP1A2 (Xu et al. 2016; Hubeny et al. 2016), indicating roles of OATP1A2 in disposition and activities of antimalarial drugs in erythrocytes. OATP1A2 is expressed in photoreceptor bodies, somas of amacrine cells, and retinal pigment epithelium, mediating the cellular uptake of all-trans-retinol (Chan et al. 2015; Gao et al. 2015), inferring implication of OATP1A2 in canonical visual cycle. The OATP1A2-mediated uptake of all-trans-retinol may be inhibited by chloroquine and hydroxychloroquine (Xu et al. 2016), which may provide novel insights into retinal dysfunction induced by certain drugs. For instance, digoxin and antimalarial drugs (chloroquine and hydroxychloroquine) may induce retinopathy (Kinoshita et al. 2014; Weleber and Shults 1981; Yaylali et al. 2013), which could plausibly be due to decrease in all-trans-retinol uptake into the retinal cells via inhibiting retinal OATP1A2 function, resulting in dysfunction of the canonical visual cycle and

toxic accumulation of retinoids. Substance P and vasoactive intestinal peptides were detected in retinal neurons expressing OATP1A2 (Gao et al. 2015), demonstrating roles of OATP1A2 in reuptake of these peptide neurotransmitters released from retinal neurons. OATP1A2 is abundantly expressed in neurons and neuronal processes, which points to the possibility that OATP1A2 could be involved in the homeostasis of neurosteroids or neuropeptides (Gao et al. 2015).

Breast cancer tissues were reported to have tenfold higher expression of OATP1A2 than surrounding normal tissues (Miki et al. 2006; Meyer zu Schwabedissen et al. 2008). OATP1A2 expression in hormone receptor-positive tumor tissues was 2.4 times greater than hormone receptor-negative tissues (Banerjee et al. 2014). Moreover, hormone-dependent breast cancer cells (MCF7) also showed higher expression of OATP1A2 protein and transport of E3S than hormone-independent breast cancer cells (MDA/LCC6-435, MDA-MB-231, and MDA-MB-468) (Banerjee et al. 2012). Malignant breast tissues showed remarkably higher the concentrations of E3S compared with surrounding normal tissues (Chetrite et al. 2000), which may be attributed to high expression of OATP1A2. In consistence with this, tumor tissues from MDA-MB-231 xenograft mice and MCF7 xenograft mice also possessed higher levels of E3S compared with blood (Banerjee et al. 2013). In addition, MCF-7 tumor tissues showed significantly higher expression OATP1A2 protein and higher accumulation of E3S than MDA-MB-231 tumor. A clinical trial (Hashimoto et al. 2014) on 124 patients receiving anthracycline/taxane-based neoadjuvant chemotherapy showed that among the 124 biopsied tumors obtained prior to chemotherapy, 44% and 52% tumors showed high levels of OATP1A2 and OCT6, respectively, and 26% had high levels of both OATP1A2 and OCT6. The tumors with a high OATP1A2/high OCT6 level showed significantly higher rates of pathologic good response and pathologic complete response than tumors with low levels of at least one of OATP1A2 and OCT6, inferring that combined high OATP1A2/high OCT6 may be a potential predictor of response to anthracycline/taxane-based chemotherapy in breast cancer, especially in triple negative tumors.

3.2.4 OATP1B3

OATP1B3 (previously known as OATP8 and LST-2), cloned by *SLCO1B3*, shares 80% amino acid sequence identity with OATP1B1 and is mainly expressed on the sinusoidal membrane of human hepatocytes. Like OATP1B1, OATP1B3 facilitates hepatic uptake of a variety of exogenous and endogenous compounds. Endogenous substrates of OATP1B3 are similar to those of OATP1B1 including bilirubin, bile acids, conjugated steroids, eicosanoids, and thyroid hormones, but CCK-8 is mainly transported by OATP1B3. OATP1B3 also accepts therapeutic drugs including atrasentan, bosentan, docetaxel, digoxin, enalapril, erythromycin, fexofenadine, fluvastatin, glucuronide imatinib, methotrexate, mycophenolic acid glibenclamide, olmesartan, ouabain, paclitaxel, pitavastatin, pravastatin, rifampicin, rosuvastatin,

rifampicin, SN-38, telmisartan, and valsartan as its substrates. It should be noted that substrates of OATP1B3 often overlap those of OATP1B1, but telmisartan seems to be preferentially transported by hepatic OATP1B3. Clarithromycin, cyclosporine, azithromycin, erythromycin, rifampicin, glibenclamide, glimepiride, nateglinide, roxithromycin, and tolbutamide were identified to be OATP1B3 inhibitor, inferring that these compounds may interact with OATP1B3 substrates (Kalliokoski and Niemi 2009; Meyer zu Schwabedissen et al. 2014).

Under normal physiological conditions, OATP1B3 is selectively expressed in human hepatocytes, but OATP1B3 is also expressed in some tumors including gastric, colon, and pancreatic cancers (Abe et al. 2001; Teft et al. 2015; Thakkar et al. 2013; Hays et al. 2013). OATP1B3 expression within colon tumors is considered to be a cancer-specific isoform (Thakkar et al. 2013). A report also showed (Teft et al. 2015) that colon tumor tissue had a significantly higher OATP1B3 expression compared with paired normal tissue and that OATP1B3 expression was associated with clinical response to CTP-11. The progression-free survival following CPT-11-based chemotherapy regimens was significantly reduced in patients with high OATP1B3 expression compared with patients with low expression, suggesting that OATP1B3 expression may correlate with poorer clinical response to CPT-11 therapy. Frequency and extent of OATP1B3 expression were dependent on stages and types of pancreatic cancer (Hays et al. 2013). Frequencies of OATP1B3 expression in normal pancreas (10%) was lower than that in cancer adjacent normal pancreas tissue (20%). High frequency of OATP1B3 expression was found in hyperplasia, pancreatic inflammation, and chronic pancreatitis specimens (38%) as well as adenosquamous carcinomas (up to 50%). Interestingly, OATP1B3 was highly expressed in pancreatic hyperplasia and early-stage adenocarcinomas with decreasing expression in higher-stage adenocarcinomas. The frequency of OATP1B3 expression was 30% in stage one pancreatic adenocarcinoma cases, decreased in stage two (18%) and stage three adenocarcinoma (13%), and was completely absent in metastatic tissues from primary pancreatic adenocarcinoma, inferring that OATP1B3 is a potential marker to diagnose patients with early-stage pancreatic adenocarcinomas (Hays et al. 2013). OATP1B3 was also expressed in the insulin-producing and insulin-secreting β-cells of human pancreas. In vitro study also demonstrated that overexpression of OATP1B3 significantly enhanced both uptake of glibenclamide and glibenclamide-induced insulin secretion in MIN6 cells (Meyer zu Schwabedissen et al. 2014). These results inferred roles of OATP1B3 in release of insulin.

3.2.5 OATP 2B1

OATP2B1 (previously OATP-B), coded by *SLCO2B1*, is most abundant in human liver and considered to be the third OATP localized to the basolateral hepatocyte membrane of human liver (Kullak-Ublick et al. 2001; Tamai et al. 2000). Its

apparent molecular mass is 84 kDa, which is reduced after deglycosylation to 60 kDa. Interestingly, the molecular mass of glycosylated protein is dependent on the type of cells. The apparent molecular sizes of OATP2B1 expressed in MCF-7 cells and HEK293 cells (Matsumoto et al. 2015) were approximately 116 kDa and 84 kDa, respectively, but deglycosylation of OATP2B1 shifted the band size to about 59 kDa in both MCF-7 and HEK293 cells. Rodent Oatp2b1 is considered to be the closest orthologue of OATB2B1, which shares 77% amino acid sequence identity with human OATP2B1 (Tamai 2012).

Unlike other OATPs, OATP2B1 shows much broader expression profiles. Northern blot analysis showed the highest level of OATP2B1 expression in the liver and in decreasing order of signal intensity in the spleen, placenta, lung, kidney, heart, ovary, small intestine, and brain (Kullak-Ublick et al. 2001). OATP2B1 protein is located at the basolateral membrane of hepatocytes, at the apical membrane of enterocytes, at the endothelium of the BBB, at the endothelial cells of the heart, at the myoepithelium of mammary ducts, and in the placenta (Obaidat et al. 2012).

OATP2B1 mediates transport of some endogenous compounds including DHEAS, E3S, PGE2, substance P, and vasoactive intestinal peptide (Tamai et al. 2000; Kullak-Ublick et al. 2001), but does not mediate transport of bile salts nor $E_2$17βG (Kullak-Ublick et al. 2001). Unlike OATP1B1, OATP2B1 accepted sulfate conjugates of steroids but not glucuronide conjugates as its substrates, whereas OATP1B1 transported both types of steroid conjugates (Tamai et al. 2001). Therapeutic drugs including atorvastatin, benzylpenicillin, bosentan, BSP, fexofenadine, fluvastatin, glibenclamide, pravastatin, rosuvastatin, and unoprostone are also OATP2B1 substrates (Grube et al. 2006; Kalliokoski and Niemi 2009). Atorvastatin, BSP, cerivastatin, cyclosporine A, glyburide, gemfibrozil, and rifamycin SV have been identified to be inhibitors of OATP2B1 (Grube et al. 2006; Kis et al. 2010). Some flavonoids (such as naringin, naringenin, hesperidin, hesperetin, phloridzin, phloretin, quercetin, and kaempferol) in fruit juices, HIV protease inhibitors (such as atazanavir, darunavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, and tipranavir), and non-nucleoside reverse transcriptase inhibitors (efavirenz) also inhibited OATP2B1-mediated transport of E3S (Kis et al. 2010; Shirasaka et al. 2013).

OATP2B1-mediated transport is substrate- and pH-specific. For example, OATP2B1-mediated transports of atorvastatin, cerivastatin, DHEAS, E3S, fexofenadine, fluvastatin, rosuvastatin, pravastatin, and pitavastatin are pH sensitive. In HEK293 cells expressing OATP2B1, it was found that acidic extracellular pH increased their uptakes compared to pH 7.4 (Varma et al. 2011; Visentin et al. 2012; Nozawa et al. 2004). The OATP2B1-mediated uptake of rosuvastatin and pravastatin at pH 5.5 was eightfold higher compared to that at neutral pH. Uptakes of E3S, DHEAS, and fexofenadine at low pH (pH = 5) were about two- or threefold higher compared with those at neutral pH (pH = 7.4). However, pravastatin, cerivastatin, pitavastatin, and TCA were not substrates of OATP2B1 at neutral pH (Varma et al. 2011; Nozawa et al. 2004). OATP2B1-mediated uptake of pemetrexed is also pH-specific. The optimal uptake occurred at pH 4.5–5.5, then falling precipitously with an increase in pH, but BSP uptake was independent of pH (Visentin et al. 2012).

On the other hand, tebipenem pivoxil was transported only under neutral or weakly alkaline conditions (Kato et al. 2010). These results indicate that contributions of OATP2B1 to the tissue disposition of its substrates are dependent on both the level of expression in tissues and the pH in the tissue microenvironment.

Tissue characteristics of OATP2B1 distribution affect disposition and pharmacological/toxic effects of its substrates. For example, vascular endothelium of human heart demonstrated significant expression of OATP2B1 mRNA (Grube et al. 2006). Furthermore, expression of OATP2B1 protein in human coronary artery smooth muscle cells (HCASMC) was significantly higher than that in human coronary artery endothelial cells (HCAEC). In accordance with this, HCASMC possessed higher accumulation of atorvastatin and showed more potently inhibitory effect of atorvastatin on cell proliferation (IC_{50} 0.62 μM) than HCAEC (IC_{50} 4.45 μM), demonstrating HCASMC-specific effect of atorvastatin (Hussner et al. 2015). Teniposide, another substrate of OATP2B1, also showed HCASMC-specific effect (Hussner et al. 2015). Generally, in-stent restenosis often results from migration and proliferation of coronary artery smooth muscle cells. Some drug-eluting stents have been shown to enable effective limitation of neointimal hyperplasia, but these drug-eluting stents may adversely affect endothelial regrowth, leading to late thrombotic events (Losordo et al. 2003). The atorvastatin and teniposide characterizes HCASMC-specific effects, indicating that the two compounds may serve as drug-eluting stent candidate drug with HCASMC-specific effects and that endogenously expressed OATP2B1 significantly influences the uptake of substrate drugs, thereby governing cell specificity (Hussner et al. 2015).

Amiodarone is an ideal antiarrhythmic agent, but there is a risk of development of life-threatening amiodarone-induced pulmonary toxicity. OATP2B1 is also expressed in the lung (Kullak-Ublick et al. 2001; Tamai et al. 2000) and in human alveolar epithelial-derived cell line A549 (Seki et al. 2009). In line with this issue, the A549 cell had high activity of amiodarone uptake, which may be inhibited by OATP2B1 siRNA or some substrates of OATP2B1 (BSP, DHEAS, and E3S), inferring role of OATP2B1 in lung accumulation of amiodarone (Seki et al. 2009).

Statins reduce risks of acute coronary syndrome, stroke, and atherosclerotic lesions partly due to affecting platelet function and inhibiting thrombus formation (Futterman and Lemberg 2004; Zhou and Liao 2010), which is partly attributed to inhibiting Ca^{2+} mobilization by thrombin stimulation (Niessen et al. 2009). OATP2B1 is highly expressed in plasma membrane fractions of platelet and megakaryocytes (Niessen et al. 2009). Coincubation with 10 μM E3S was reported to attenuate the atorvastatin-mediated inhibition of Ca^{2+} mobilization, demonstrating roles of OATP2B1 in inhibition of platelet function by atorvastatin (Niessen et al. 2009). Besides statins, some drugs and metabolites such as DHEAS, fexofenadine, or E3S could be taken up into platelets by this transporter. For example, DHEAS was reported to inhibit in vitro thrombin-dependent platelet aggregation in a dose-dependent manner (Bertoni et al. 2012). Clinical trial (Martina et al. 2006) showed that short-term treatment with DHEAS increased platelet cGMP production in healthy elderly subjects, whereas treatment with E3S showed an inhibitory effect on both thrombin-induced platelet aggregation and 5-HT releases (Blache et al. 1995).

OATP2B1 is highly expressed in human placenta. Interestingly, expression of OATP2B1 significantly correlates to expression of BCRP in human placenta. In MDCK-expressing both OATP2B1 and BCRP, it was found that basal-to-apical transports of both E3S and DHEAS were significantly increased without affecting transport in the apical-to-basal direction, demonstrating the potential for a functional interaction of OATP2B1 and BCRP in transepithelial transport of steroid sulfates in human placenta (Grube et al. 2007). Clinical samples also demonstrated that to be compared with normal tissues, +malignant tumor showed significantly higher level of OATP2B1 mRNA and that the expression of OATP2B1 mRNA was significantly correlated with histological grade of tumor, Ki-67 labelling index, and mRNA expression of steroid sulfatase. Moreover, the expression level of OATP2B1 mRNA in luminal B-like cancers was higher than that in luminal A-like cancers. Cell-based assays showed that the stimulatory effects of E3S on cell proliferation were significantly greater in MCF-7 cells overexpressing OATP2B1 than that in control cells. And BSP blocked E3S-stimulated proliferation of MCF-7 cells. These findings demonstrated a significant role of OATP2B1-mediated E3S uptake in breast cancer cells, inferring that OATP2B1 may be useful as a novel target or biomarker for estrogen receptor breast cancer (Matsumoto et al. 2015), although other OATPs may be involved in uptake of E3S.

Expression of OATP2B1 in intestinal brush-border membranes plays an important role in the gastrointestinal absorption of therapeutic drugs. Several studies have demonstrated that some fruit juices decrease plasma exposure of OATP2B1 substrate drugs via inhibiting intestinal OATP2B1 when they are coadministrated. For example, coadministration (Tapaninen et al. 2011) of organ juice or apple juice reduced aliskiren C_{max} by 80% and 84% and AUC^{0-72} by 62% and 63%, respectively. Intestinal OATP2B1 was also reported to contribute to gastrointestinal toxicity of SN-38, inferring that coingestion of apple juice may prevent gastrointestinal toxicity of SN-38 in mice caused by oral administration (Fujita et al. 2016).

3.2.6 Other OATPs

OATP1C1 (OATP-F), a 712-amino acid protein, is mainly expressed in the human brain, testis, and ciliary body and shows a high affinity to thyroid hormones; thus it may play an important role in the delivery and disposition of thyroid hormones in the brain and testis (Mayerl et al. 2014). OATP2A1 (PGT), a protein of 643 amino acids, is broadly expressed in different tissues and acts as a prostaglandin transporter, where it mediates the distribution of prostanoids, such as PGE_2, $PGF_{2\alpha}$, PGD_2, and TXB_2, and implicates in diverse physiological and pathophysiological processes in many organs (Nakanishi and Tamai 2017). OATP3A1 (OATP-D) is expressed in two splice variants OATP3A1_v1 and OATP3A1_v2. OATP3A1_v2 lacks 18 amino acids at the COOH-terminal end (692aa) compared with OATP3A1_v1 (710aa). OATP3A1_v1 is ubiquitously expressed including the heart, whereas OATP3A1_v2 is predominantly expressed in the testis and brain. OATP3A1

transports E3S, PGE2, thyroxine, vasopressin, and benzylpenicillin (Huber et al. 2007). OATP4A1 (OATP-E) is ubiquitously expressed, and it transports estrogens, PGs, thyroid hormones, taurocholate, benzylpenicillin, and unoprostone. OATP4A1 is also expressed in colorectal cancer, which mediate E3S uptake into cells (Gilligan et al. 2017). Epidemiological studies indicate that 17β-estradiol prevents colorectal cancer (Rawłuszko-Wieczorek et al. 2015). The significantly increased OATP4A1 mRNA levels were found in cancerous tissue (Rawłuszko-Wieczorek et al. 2015) and the percentage of OATP4A1-positive cells was also significantly higher in tumor and mucosal cells adjacent to the tumor compared to the mucosa of nonmalignant samples. Importantly, OATP4A1 abundance was negatively associated with tumor recurrence in early-stage colorectal cancer (Buxhofer-Ausch et al. 2018). OATP4C1 (OATP-H) is localized to the basolateral membrane of human proximal tubule cells, and therefore it may mediate the uptake of its substrates from the blood into the kidney. OATP4C1 transports thyroid hormones, digoxin, methotrexate, and the antidiabetic drug sitagliptin; some drugs induced drug-drug interaction via affecting renal OATP4C1 (Sato et al. 2017). OATP5A1 (OATP-J) is known only at the cDNA level, and mRNA of OATP6A1 (OATP-I) has been detected in the testis (Kalliokoski and Niemi 2009).

3.3 Organic Cation Transporters (OCTs) and Organic Cation/Carnitine Transporters (OCTNs)

3.3.1 General Properties and Distribution of OCTs/OCTNs

The body is equipped with broad-specificity transporters for the uptake, elimination, and distribution of endogenous organic cations, cationic drugs, and toxins. These transporters belong to the SLC22 family and the MATE family. The SLC22 family contains the three subtypes: the electrogenic organic cation transporters (OCT1, OCT2, and OCT3), the electroneutral organic cation transporters (OCTN1, OCTN2, and OCTN3), and the carnitine/cation transporter OCT6 (Koepsell et al. 2007; Zhou et al. 2017). OCT1 and OCT2 have 70% amino acid identity to each other and approximately 50% identity with OCT3. OCTN1 and OCTN2 share 77% identity with each other and 31~37% identity with OCT1-3. OCT6 has 36%, 38%, and 37% identity to OCT1, OCT1, and OCTN2, respectively (Enomoto et al. 2002).

OCT and OCTN proteins contain between 543 and 557 amino acids. They are predicted to contain 12 TMDs with intracellular amino and carboxy-termini (Fig. 3.5). A large extracellular loop between TMD1 and TMD2 contains potential N-glycosylation sites, and a large intracellular loop between TMD6 and TMD7 contains multiple putative phosphorylation sites (Jonker and Schinkel 2004; Roth et al. 2012; Koepsell et al. 2007). The N-glycosylation sites in extracellular loop may be involved in protein stability, intracellular routing, or protection from extracellular proteases (Jonker and Schinkel 2004).

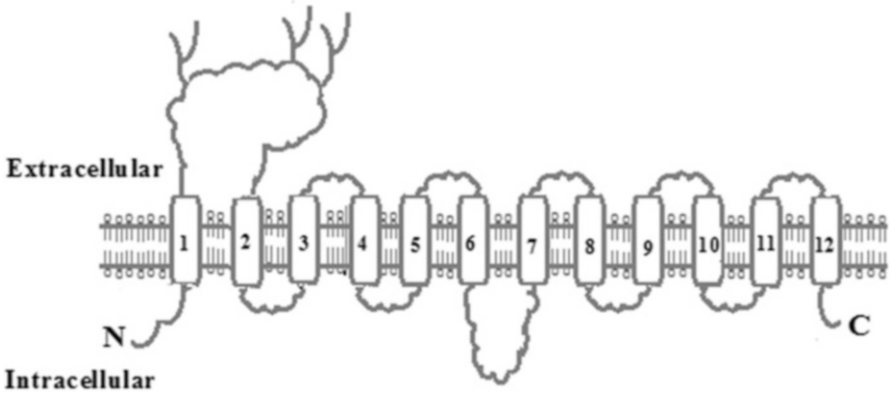

Fig. 3.5 Predicted secondary structure of OCTs/OCTNs. The proteins are thought to contain 12 TMDs with both N- and C-terminus located intracellularly. The first large extracellular loop contains three putative N-linked glycosylation sites (indicated by "Y")

3.3.1.1 OCT1

Human OCT1 and other species Oct1 are coded by *SLC22A1* and *Slc22a1*, respectively. Human OCT1 is mainly expressed in the sinusoidal membrane of the hepatocytes and is considered to be a liver-specific transporter, along with OATP1B1 and OATP1B3 (Koepsell et al. 2007; Lozano et al. 2013; Nies et al. 2009), whereas in rodents, Oct1 is strongly expressed in the liver, kidney, and small intestine (Koepsell et al. 2007; Jonker and Schinkel 2004), indicating that tissue distribution of human OCT1 differs from rodent Oct1. OCT1/Oct1 is located in the luminal membrane of epithelial cells in the trachea and bronchi of human, rat, and mouse (Lips et al. 2005), mediating release of acetylcholine in the respiratory epithelium (Fig. 3.6).

OCT1 is also expressed in many other organs including the spleen, skin, skeletal muscle, heart, brain, stomach, placenta, cholangiocytes, and epithelial cells (Koepsell et al. 2007; Zhou et al. 2017; Lozano et al. 2013). In rat kidney, Oct1 is located to the basolateral membrane of epithelial cells in the S1 and S2 segments of proximal tubules. In human kidney, OCT1 is also demonstrated at the apical membrane of epithelial cells in the proximal and distal tubules of the nephron (Lozano et al. 2013), but extent is less than that in rodent kidney. OCT1 and OCT2 were also detected in $CD4^+$ cell from patients with HIV-1 infection. The intracellular concentration of lamivudine and its active metabolite lamivudine triphosphate were positively related to expression of OCT1 and OCT2 mRNA, indicating a role of OCT1 and OCT2 for the cellular accumulation of lamivudine in HIV-infected individuals (Jung et al. 2013).

Over 200 *SLC22A1* SNPs have been identified. The OCT1 variants *R61C* (*c.181C>T*), *C88R* (*c.262T>C*), *G220V* (*c.659G>T*), *P341L* (*c.1022C>T*), *G401C* (*c.1203G>A*), *G465* (*c.1393G>A*), *P283L* (*c.848C>T*), *S189L* (*c.566C>T*), *420del* (*1260delATG*), and *C465R* (*c.1393G>A*) were associated with reduced or lost uptake of 1-methyl 1-4-phenylpyridinum or metformin in vitro (Zaïr et al. 2008). It is a

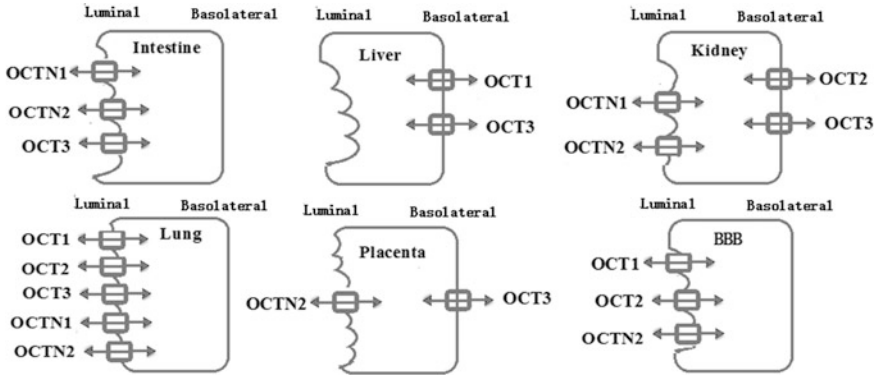

Fig. 3.6 Expression of OCTs/OCTNs in human epithelial cells of the intestine, liver, kidney, lung, placenta, and brain endothelial cells (BBB)

remarkable that striking differences exist in the frequency of loss of OCT1 activity worldwide. Most East Asian and Oceanian individuals have completely functional OCT1, but 80% of native South American Indians lack functional OCT1 alleles (Seitz et al. 2015).

3.3.1.2 OCT2

Human OCT2 and animal Oct2 are coded by *SLC22A2* and *Slc22a2*, respectively. Compared with other OCTs, human OCT2 has a more restricted expression pattern. OCT2 is most strongly expressed in the kidney not in the liver (Lozano et al. 2013); thus OCT2 is generally considered to be a kidney transporter (Roth et al. 2012). OCT2 is also expressed in other organs including the small intestine, lung, skin, brain, and choroid plexus (Koepsell et al. 2007). In human kidney, OCT2 is expressed in all three segments of proximal tubules, whereas in rat kidney, Oct2 is localized to the S2 and S3 segments. OCT2 is localized to the basolateral membrane of epithelial cells in renal proximal tubules and small intestine, but to the luminal membrane of epithelial cells in trachea and bronchi (Fig. 3.6). In human brain OCT2 is expressed in neurons, in the luminal membrane of endothelial cells of microvessels, and in the apical membrane of epithelial cells in choroid plexus (Koepsell et al. 2007; Koepsell 2013).

3.3.1.3 OCT3

Human OCT3 and animal Oct3 are coded by *SLC22A3* and *Slc22a3*, respectively. OCT3, also known as the extraneuronal monoamine transporter, has the widest

tissue distribution of the OCTs. The strongest expression of human OCT3 mRNA was found in the skeletal muscle, liver, placenta, and heart. In rodents, expression of Oct3 is also demonstrated in additional organs, Sertoli cells, and basophile granulocytes. OCT3 protein is localized to the basolateral membrane of the trophoblast in the placenta, to the sinusoidal membrane of hepatocytes, to the basolateral membrane of epithelial cells of renal proximal tubules, and to luminal membranes of bronchial epithelial cells and small intestinal enterocytes (Roth et al. 2012; Koepsell et al. 2007) (Fig. 3.6). Oct3 is also expressed in the hippocampus, area postrema, subfornical organ, medial hypothalamus, and ependym of the third ventricle of rodents.

3.3.1.4 OCTN1

Human OCNT1 and animal Ocnt1 are coded by *SLC22A4* and *Slc22a4*, respectively. OCTN1 is mainly expressed in epithelial and muscle cells. In human, the strongest expression is demonstrated in the kidney, skeletal muscle, bone marrow, and trachea. OCTN1 mRNA is also detected in $CD68^+$ macrophages, $CD43^+$T cells, $CD14^+$ mononuclear cells, and mitochondria (Fig. 3.6). Mouse Octn1 is localized to the apical membrane of cortical proximal tubular epithelial cells. Rodent Octn1 is also expressed in additional organs and in Sertoli cells (Roth et al. 2012; Koepsell et al. 2007; Koepsell 2013).

3.3.1.5 OCTN2

Human OCTN2, coded by *SLC22A5*, is a high-affinity Na^+-L-carnitine cotransporter and has a relatively ubiquitous distribution. The strongest expression of OCTN2 is demonstrated in the liver, kidney, skeletal muscle, heart, and placenta. OCTN2 protein is localized to the brush-border membrane of renal proximal tubule cells and small intestinal enterocytes (Roth et al. 2012; Koepsell et al. 2007; Koepsell 2013) (Fig. 3.6).

3.3.1.6 OCT6

Human OCT6, coded by *SLC22A16*, is mainly expressed in plasma membranes of Sertoli cells and in the luminal membrane of epithelial cells in the epididymis. In addition, OCT6 is detected in the embryonic liver, hematopoietic cells, leukemias, skeletal muscle, kidney, liver, placenta, mammary gland, and brain. OCT6 is a high-affinity L-carnitine transporter that also translocates various organic cations such as spermidine, doxorubicin, and bleomycin A5 (Koepsell 2013).

3.3.2 Substrates and Inhibitors of OCTs/OCTNs

The basic transport characteristics of OCT1, OCT2, and OCT3 are similar. First, OCTs translocate a variety of organic cations with widely differing molecular structures (Zhou et al. 2017); thus they are also defined as "polyspecific" organic cation transporters. The relative molecular mass of most compounds transported by OCT1-3 is below 500, and the smallest diameter of the molecules is below 4 Å. They have extensively overlapping substrate specificities. Their substrates include the model substrate tetraethylammonium, the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+), clinically used drugs such as antiparkinsonians (amantadine and memantine), antidiabetics (metformin), the H_2 receptor antagonists (cimetidine and ranitidine), biogenic amines (dopamine, norepinephrine, and serotonin), and some endogenous compounds (such as choline, creatinine, and prostaglandins) (Table 3.4). Compound MPP^+ is common substrates of OCT1, OCT2, and OCT3, which is frequently used as model substrate. Second, OCTs electronically translocate organic cations (Koepsell 2013; Koepsell et al. 2007). Third, OCTs operate independently of Na^+ and proton gradients. However, affinity for certain substrates depends on their degree of ionization, leading to increased transport of those substrates at reduced pH. Fourth, OCTs are able to translocate organic cations across the plasma membrane in either direction. Most substrates translocated by the OCTs are organic cations and weak bases that are positively charged at physiological pH, but noncharged compounds (such as cimetidine) or anions may be also transported.

A variety of compounds including cations (such as tetrapentylammonium, decynium-22, and disprocynium), noncharged compounds (such as corticosterone,

Table 3.4 Substrates of functionally characterized human transporters of the SLC22-family. (Boxberger et al. 2014; Tzvetkov et al. 2013; Chen et al. 2014; Hucke and Ciarimboli 2016; Andreev et al. 2016)

Transporter	Substrates
OCT1	Acyclovir, agmatine, berberine, daunorubicin, dopamine, furamidine, ganciclovir, imatinib irinotecan, lamivudine, metformin, morphine, norepinephrine, oxaliplatin, paclitacel, paclitaxel, pentamidine, picoplatin, serotonin, thiamine, vandetanib
OCT2	Acetylcholine, agmatine, amantadine, amiloride, berberine, cimetidine, cisplatin, dopamine, epinephrine, famotidine, histamine, ifosfamide, lamivudine, memantine, metformin, norepinephrine, oxaliplatin, putrescine, picoplatin, ranitidine, serotonin, zalcitabine
OCT3	Agmatine, epinephrine, etilefrine, histamine, lamivudine, lidocaine, metformin, norepinephrine, quinidine, oxaliplatin, vincristine
OCTN1	Acetylcholine, betonicine, L-carnitine, doxorubicin, ergothioneine, gabapentin, glycinebetaine, ipratropium, mitoxantrone, pyrilamine, quinidine, stachydrine, tiotropium, verapamil
OCTN2	L-carnitine, choline cephaloridine, emetine, ipratropium, mildronate, oxaliplatin pyrilamine, spironolactone, tiotropium verapamil
OCT6	L-carnitine, choline, cephaloridine, doxorubicin, emetine, ipratropium, mildronate, oxaliplatin, pyrilamine, spironolactone, tiotropium, verapamil

deoxycorticosterone, and β-estradiol), and anions (such as probenecid and α-ketoglutarate) inhibit OCT transport activity, although some of them are not transported by OCTs. The substrate and inhibitor specificities of OCT1, OCT2, and OCT3 broadly overlap, but there still exist differences in specificity between individual subtypes and between identical subtypes of different species. For example, tetraethylammonium is served as model substrate for OCT1 and OCT2, but this compound is not suitable for OCT3. Tetrabutylammonium is transported by human OCT1, human OCT2, and rabbit Oct1, but is a non-transported inhibitor of rat Oct1, mouse Oct1, and rat Oct2. Ciprofloxacin, fleroxacin, gatifloxacin, levofloxacin, lomefloxacin, moxifloxacin, norfloxacin, ofloxacin, pefloxacin, prulifloxacin, rufloxacin, and sparfloxacin significantly inhibited human OCT1 but not Oct2 function. Among the tested fluoroquinolones, only moxifloxacin inhibited human OCT3 function (Mulgaonkar et al. 2013). OCT1 is more sensitive to amprenavir, lopinavir, indinavir, nelfinavir, ritonavir, tipranavir, and saquinavir than OCT2 and OCT3. Of particular note, only ritonavir shows inhibition toward OCT2 and OCT3 (Duan et al. 2015). Tyrosine kinase inhibitors (such as imatinib, dasatinib, nilotinib, gefitinib, and sunitinib) are potent inhibitors of OCTs, with IC_{50} values generally in the low micromolar range on metformin transport. Comparison of the unbound clinically relevant peak concentrations [I] and their IC_{50} values against human OCTs showed that imatinib (for OCT1), nilotinib (for OCT3), and erlotinib (for OCT1) exert potent inhibitory effects, whose [I]/IC_{50} values were larger than 0.1 (Minematsu and Giacomini 2011). However, nilotinib and erlotinib exert less potent inhibition on mouse Oct3 and mouse Oct1 than that on human OCTs, inferring striking species differences in comparison to the human orthologues (Minematsu and Giacomini 2011). Pazopanib is also a potential inhibitor of OCT2 at the low micromolar range on uptake of cisplatin in HEK cell expressing OCT2. In keeping, coincubation of cisplatin with pazopanib led to increase in EC_{50} values of cisplatin by 2.7 times of cisplatin alone, reaching about the same values in empty vector-HEK cells (Sauzay et al. 2016).

Some inhibitors of OCTs show differences in species among individual subtypes and among identical subtypes of different species (Koepsell et al. 2007). For example, IC_{50} values (μM) of phencyclidine (4.4 for OCT1, 25 for OCT2, and 330 for OCT3), diphenhydramine (3.4 for OCT1, 14 for OCT2, and 695 for OCT3), prazosin (1.8 for OCT1, >100 for OCT2, and 13 for OCT3), citalopram (2.8 for OCT1, 21 for OCT2, and 159 for OCT3), and atropine (1.2 for OCT1, 29 for OCT2, and 466 for OCT3) show the stronger inhibition on human OCT1 than OCT2 or OCT3. On the contrast, corticosterone shows stronger inhibition on human OCT3 (IC_{50}: 0.12~0.29 μM) than OCT1 (IC_{50}: 7~22 μM) or OCT2 (IC_{50}: 34 μM), indicating that these compounds may be used to differentiate substrate transport mediated by OCT1, OCT2, or OCT3. Similarly, memantine (IC_{50}: 1.7 μM for Oct1, 73 μM for Oct2, and 295 μM for Oct3, respectively), estradiol (IC_{50}: 35 μM for Oct1, 85 μM for Oct2, and 1.1 μM for OCT3, respectively), and serotonin (IC_{50}: 38 μM for Oct1, 3600 μM for Oct2, and 970 μM for Oct3, respectively) may be used to identify uptake by rat Oct1, rat Oct2, and rat Oct3.

OCTN1, OCTN2, and OCT6 are all cation and carnitine transporters. OCTN1 transport is dependent on both sodium and proton gradients, which transports acetylcholine, L-carnitine, as well as therapeutic drugs such as quinidine, pyrilamine, verapamil, ipratropium, tiotropium, mitoxantrone, doxorubicin, and gabapentin (Koepsell 2013). OCTN2, operating as polyspecific, sodium-dependent, or sodium-independent cation transporter, transports cations such as acetyl-L-carnitine, buturyl-L-carnitine, pivaloylcarnitine, valproylcarnitine, mildronate, cephaloridine, emetine, choline, pyrilamine, verapamil, spironolactone, oxaliplatin, ipratropium, and tiotropium (Koepsell 2013; Koepsell et al. 2007). Tetraethylammonium, a substrate of both OCTN1 and OCTN2, is frequently used as a model substrate. OCTN2 appears to have different binding sites for tetraethylammonium and L-carnitine. OCT6 has a much more limited substrate specificity than other organic cation transporters (Roth et al. 2012).

3.3.3 Pharmacological and Physiological Function of OCTs/OCTNs

3.3.3.1 Impact of OCTs/OCTNs on Hepatic Drug Levels and Action

OCT1, as a main organic cation transporter in the liver, is responsible for the uptake of endogenous compounds and therapeutic drugs, showing their pharmacological and physiological function in hepatocytes. OCT1 has been identified to serve as a high-capacity thiamine transporter (Chen et al. 2014, 2015b), indicating that OCT1 may be involved in hepatic steatosis via modulating levels of thiamine. Compared with wild-type mice (Chen et al. 2014), *Slc22a1*$^{-/-}$ mice showed significant decreases in levels of thiamine metabolites, thiamine monophosphate, and thiamine pyrophosphate in the liver, accompanied by an increase in phosphorylation of AMPK and substantial decrease in liver triglyceride levels. In consistence, thiamine deficiency enhanced the phosphorylation of AMPK and reduced both hepatic triglyceride levels and ratio of liver to body weight. Conversely, human OCT1 transgenic mice showed significantly higher triglyceride concentrations, enlarged liver sizes, and enhanced mRNA and protein levels of key metabolic enzymes for lipogenesis. Both metformin treatment and Oct1 deletion had similar effects on glycolysis, lipogenesis, and energy status of hepatocytes and on key enzymes for lipogenesis. Moreover, metformin treatment reduced systemic and tissue levels of thiamine due to inhibiting Oct1 activity, partly contributing to beneficial effects on hepatic steatosis.

The liver has been established as a key organ in the elimination of monoamine neurotransmitters from the body, which is responsible for the removal of up to 70% of the serotonin from portal blood (Tyce 1990). Liver OCT1 mediates uptakes of serotonin, dopamine, and norepinephrine, which may be inhibited by diphenhydramine, fluoxetine, imatinib, and verapamil (Boxberger et al. 2014). The inhibition of serotonin uptake in the liver may lead to potential increases in circulating

serotonin levels and liver extracellular serotonin concentrations. Thus, inhibition of OCT1-mediated serotonin uptake is involved in important physiologic consequences. For example, *OCT1 rs2282143* variant (T) and *rs683369* variant (G) were reported to associated with jaundice-type progression of primary biliary cirrhosis (Ohishi et al. 2014). Mouse experiment also showed that inhibition of Oct1-medaited serotonin transport potentiated the pathogenesis of nonalcoholic steatohepatitis (Nocito et al. 2007).

OCT1 plays an important role in the uptake of drugs by hepatocytes. An important example is metformin, a widely used for the treatment of type 2 diabetes. OCT1 is mainly responsible for the uptake of metformin in the liver (Shu et al. 2007). Metformin exerts its main pharmacological activity via decreasing gluconeogenesis in the liver, resulting in a reduction in blood glucose levels. A report (Shu et al. 2007) showed that Oct1 deletion caused a reduction in the effects of metformin on AMPK phosphorylation and gluconeogenesis in mouse hepatocytes, accompanied by significant decrease in metformin uptake. Similarly, OCT inhibitor quinidine also could decrease the phosphorylation of AMPK and acetyl-CoA carboxylase by metformin in wild-type hepatocytes. Mouse experiment showed that Oct1 deficiency completely abolished the glucose-lowering effects of metformin, which was in line with significant decreases in hepatic accumulation of metformin. Interestingly, ethynylestradiol-induced cholestasis in rats showed significant decreases in mRNAs of hepatic Oct1. In line with this issue, suppression of glucagon-stimulated glucose production, stimulation of AMPK activation in hepatocytes, and glucose-lowering effect by metformin in the experimental rats were almost diminished (Jin et al. 2009). The liver is the key organ responsible for the lactic acidosis induced by metformin. Metformin was reported to significantly increase the blood lactate concentration in the wild-type mice, but only slight in *Slc22a1*$^{-/-}$ mice (Wang et al. 2003), demonstrating role of Oct1 in lactic acidosis induced by metformin.

In human, rifampin was reported to enhance glucose-lowering action of metformin, which may be partly attributed to increased OCT1 expression and hepatic uptake of metformin (Cho et al. 2011). On the contrast, coadministration of OCT1 inhibitor verapamil was reported to remarkably decrease the glucose-lowering effect of metformin without affecting plasma exposure of metformin in human (Cho et al. 2014). Five common loss-of-function OCT1 variants Arg61Cys, Cys88Arg, Gly401Ser, and Gly465Arg and a *Met420* deletion have been identified in Caucasian (Kerb et al. 2002). A large cohort of metformin-treated T2D patients (Dujic et al. 2015) showed that patients carrying two OCT1 reduced-function alleles had more than twice the odds of intolerance compared to one or no deficient allele carriers. Moreover, individuals with two reduced-function OCT1 alleles who were treated with OCT1 inhibitors were over four times more likely to develop intolerance. Interestingly, patients treated with verapamil had seven times higher odds of developing metformin intolerance (Dujic et al. 2015). Similarly, individuals carrying at least one reduced-function variant of OCT1 were reported to show significantly lower effects of metformin in glucose tolerance tests than reference OCT1 alleles (Shu et al. 2007). In addition, patients resistant to metformin were reported to show higher frequency of OCT1 *rs622342AC* or CC genotype than patients responsive to

metformin (Umamaheswaran et al. 2015). Further analysis showed that the decreases in glucose-lowering action of metformin induced by reduced-function OCT1 were attributed to decreases in liver distribution of metformin (Sundelin et al. 2017).

Another example is O-desmethyltramadol, the main active metabolite of tramadol, which is produced in the liver via demethylation by CYP2D6. O-desmethyltramadol, but not tramadol, is an OCT1 substrate (Tzvetkov et al. 2011). Clinical report showed that the individuals carrying loss-of-function OCT1 variants had significantly higher plasma concentrations of O-desmethyltramadol and significantly prolonged miosis, a surrogate marker of opioidergic effects (Tzvetkov et al. 2011). Moreover, *AUC* of (+)-O-desmethyltramadol were reported to be negatively correlated to numbers of active OCT1 alleles. Loss of OCT1 function resulted in reduced tramadol consumption in patients recovering from surgery (Stamer et al. 2016). Hepatic uptake of morphine is also mediated by OCT1, which was also abolished by common loss-of-function polymorphisms and strongly inhibited by irinotecan, verapamil, and ondansetron. In accordance, carriers of loss-of-function OCT1 variants showed 56% higher *AUC* of morphine following codeine administration than non-carriers (Tzvetkov et al. 2013).

OCT1 also mediates drug-induced hepatotoxicity. Some natural compounds such as monocrotaline (Tu et al. 2013), nitidine chloride (Li et al. 2014), and retrorsine (Tu et al. 2014) are substrates of OCT1, inferring that hepatic OCT1 plays an important role in drug-induced hepatotoxicity together with CYP450. OCT1 also mediates hepatic uptake of cis-diammine(pyridine)chloroplatinum (Li et al. 2011). In mice, Oct1 deletion or coadministration of disopyramide decreased platinum-DNA adduct formation in the liver and drug-induced hepatotoxicity, which was in line with lower concentration of cis-diammine(pyridine) chloroplatinum. Contrarily, *Oct1* deletion enhanced other toxicity of the compound, including body weight loss, renal toxicity, and hematological toxicity due to high plasma exposure (Li et al. 2011).

3.3.3.2 Impact of OCTs on Kidney Drug Levels and Action

Human OCT2 is highly expressed kidneys in the basolateral membrane of proximal tubule cells, which is responsible for both drug accumulation in kidney and excretion into urine. A typical example is cisplatin. Accumulative evidences have demonstrated that OCT1 and OCT2 have been implicated in the cellular uptake of cisplatin and its nephrotoxicity. Clinical reports showed that OCT2 variant *rs316019* (*c.808G>T*) or coadministration of OCT2 inhibitors (cimetidine and verapamil) ameliorated cisplatin-induced nephrotoxicity without altering exposure of cisplatin (Filipski et al. 2009; Iwata et al. 2012; Sleijfer et al. 1987; Sprowl et al. 2013; Zhang and Zhou 2012). In mice, Oct1/Oct2 deficiency or coadministration of Oct inhibitors (cimetidine, imatinib, or cimetidine) protected from cisplatin-induced nephrotoxicity (Ciarimboli et al. 2010; Filipski et al. 2009; Franke et al. 2010; Sprowl et al. 2014; Tanihara et al. 2009). Pharmacokinetic data showed that deletion of Oct1/Oct2 significantly impaired urinary excretion of cisplatin without affecting plasma levels

of platinum (Filipski et al. 2009; Sprowl et al. 2014). Mouse Oct1, Oct2, and Mate1 mediate cisplatin transport (Nakamura et al. 2010), but only rat Oct2 mediates cisplatin transport (Yokoo et al. 2007). The strong uptake of cisplatin by OCT2 and weak tubular secretion into the urine by MATEs in common contribute to high renal accumulation of cisplatin, leading to cisplatin nephrotoxicity (Yokoo et al. 2007). Oxaliplatin, another platinum agent, is transported by both rat OCT2 and rat Mate1 (Yokoo et al. 2007). In humans, MATE1, MATE2-K, and OCT2 also mediate the transport of oxaliplatin (Yonezawa et al. 2006). These findings may explain that oxaliplatin uptake into the kidney is mediated by rat OCT2 and human OCT2, but it is a weakly nephrotoxic agent in rats and humans.

Nephrotoxicity is a relevant limitation of gentamicin, and obese patients were associated with an increased risk for gentamicin-induced kidney injury (Corcoran et al. 1988). Animal experiments showed that compared with lean rats, obese rats sustained more severe nephrotoxicity of gentamicin (Corcoran and Salazar 1989), which was in line with substantially higher levels of gentamicin in the kidney (Salazar et al. 1992). Feeding with high-fat diet was reported to increase levels of both Oct2 and Oct3 mRNA and protein in the kidney of mice (Gai et al. 2016). Increased expressions of OCT2 and OCT3 were detected in kidney biopsy specimens of obese patients (Gai et al. 2016). In vitro study confirmed that gentamicin was transported by human OCT2 not OCT1 or OCT3 (Gai et al. 2016). The increased expression of kidney OCT2 may explain the higher renal accumulation of gentamicin, thereby conferring an increased risk of renal toxicity in obese patients.

Another example is metformin. The drug is transported into proximal tubule cells by OCT2 as well as OCT1 and then effluxed into the urine by renal MATE1. Metformin is a superior substrate for renal OCT2 rather than hepatic OCT1, indicating dominant roles of renal OCT2 in metformin pharmacokinetics (Kimura et al. 2005). In normal healthy subjects, renal clearance of metformin is much greater than glomerular filtration rate and shows considerable inter-individual variability (Yin et al. 2006), ranging from 300 to 1000 mL/min, which may come from genetic component. Four polymorphic nonsynonymous variants of OCT2 (*M165I*, *A270S*, *R400C*, and *K432Q*) have been identified, of which *A270S* (*c.808G>T*) (*rs316019*), p.270Ala>Ser was widely investigated. However, effects of this variant on uptake of metformin are often confused. For example, uptake of metformin in HEK-293 cells expressing OCT2-808T was reported to be similar to cells expressing OCT2-808G, but the OCT2-808T variant significantly impaired uptake kinetics of MPP^+, dopamine, norepinephrine, and propranolol, inferring substrate-dependent characteristics (Zolk et al. 2009). Similarly, variant of OCT2-808T was reported to decrease the transport activity of metformin in oocytes expressing OCT2 (Song et al. 2008). On the contrast, HEK-293 cells expressing OCT2-808T had significantly higher uptake of metformin than cells expressing OCT2-808G (Chen et al. 2009a). Similar confused results have been demonstrated in clinical trials. A clinical report demonstrated that association of OCT2 *c.808G>T* variant with a reduced renal clearance of metformin, whose renal clearances were GG>GT>TT genotype in Chinese subjects (Wang et al. 2008). Moreover, coadministration of cimetidine decreased metformin

clearance, but the extent to decrease was significantly lower patients carrying TT genotype than GG genotype of *c.808G>T* (Wang et al. 2008). Carriers with genotype (GT) of *c.808 G>T* showed stronger glucose-lowering efficiency of metformin than those with the wild-type homozygote (Hou et al. 2015b), and the patients with genotype (TT) of c.808G>T had a higher incidence of hyperlactacidemia following administration of metformin than GG genotype (Li et al. 2010). However, in Caucasian and African American ancestries, it was reported that subjects carrying GT genotype of *c.808G>T* had significantly higher renal clearance and the net secretion of metformin than GG genotype (Chen et al. 2009a). It should be noted that in addition to OCT2 gene, other transporters (such as OCT1 and MATEs) may affect metformin renal elimination. For instance, in 50 Caucasian volunteers (Christensen et al. 2013), it was reported that alone polymorphism of *c.808G>T* little affected the renal and the secretory clearance of metformin. Both renal and the secretory clearance were significantly increased for the volunteers with *c.808G>T* who were also homozygous for the MATE1 reference variant *g.−66T>C*. In the volunteers with *c.808G>T* who were also heterozygous for *g.−66T>C*, both renal and the secretory clearance were significantly reduced when compared with volunteers with *c.808G>T* carrying the *g.−66T>C* reference genotype, demonstrating counteracting effects of the *c.808G>T* and *g.−66T>C* on the renal elimination of metformin (Christensen et al. 2013).

One important substrate of OCT2 is creatinine (Urakami et al. 2004; Ciarimboli et al. 2012), although other transporters such as MATE1, MATE2-K, and OAT2 also mediate excretion of creatinine into urine. Serum creatinine or creatinine clearance is commonly used as a marker of renal function, but creatinine clearance usually exceeds the glomerular filtration rate because of the tubular secretion of creatinine. Importantly, sometimes, alterations in serum creatinine and creatinine clearance may not reflect changes in glomerular filtration rate. For example, *Slc22a1/2*$^{-/-}$mice showed significant lower creatinine clearance than wild-type mice, accompanied by significant decreases in kidney/serum ratio of creatinine levels (Ciarimboli et al. 2012). Patients carrying the G allele of *rs2504954* showed higher serum creatinine levels compared with the T allele (Ciarimboli et al. 2012). In addition, an acute elevation of serum creatinine in human by cisplatin or INCB039110 may be partly attributed to inhibition of OCT2 activity (Ciarimboli et al. 2012; Zhang et al. 2015).

3.3.3.3 OCTs and Brain

OCT1 and OCT2 are located in microvessel endothelial cells of the brain, showing their roles in BBB. Proteins and mRNA of OCT1 and OCT2 were detected in brain microvessels endothelial cells of C57BL/6 mice and rats (Wu et al. 2015a; Lin et al. 2010); however, their expressions were reduced in aged mice (Wu et al. 2015a). N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is an important neurotoxin used for establishing animal models of Parkinson's disease. The compound passes readily across BBB and is then converted intracerebrally to neurotoxin 1-methyl-4-phenylpyridine (MPP^+). MPTP transport across BBB is partly mediated by OCT1

and OCT2. Silencing of OCT1, OCT2, and both or coadministration of OCT1/2 inhibitor amantadine inhibited MPTP uptake (Lin et al. 2010; Wu et al. 2015a). In mice, knockout of *Slc22a1/a2* gene or coadministration of amantadine protected from MPTP-induced neurotoxicity, which was line with lower extracellular levels of MPTP and MPP^+ in mice. However, intrastriatal infusion of low-dose MPTP caused more severe dopaminergic toxicity in both aged mice and *Slc22a1/a2*$^{-/-}$ mice, which may be attributed to impaired BBB efflux of MPTP/MPP^+. Interestingly, mRNA of Oct1 and Oct2 were negligible in brain microvessels endothelial cells of Swiss mice (Wu et al. 2015a). These observations may partly explain that C57BL/6 mice are sensitive but Swiss mice were resistant to the neurotoxicity caused by MPTP (Hamre et al. 1999). Expression of OCT1 and OCT2 in BBB may affect transport of substrate drugs. Clinical report showed that the C variant allele of *rs622342* in OCT1 was associated with higher prescribed doses of anti-Parkinsonian drugs and shorter survival time after start of levodopa therapy. The patients with the AC or CC genotype of *rs622342* have less response to these drugs and more severe symptoms, resulting in a shorter survival period (Becker et al. 2011).

OCT2 and OCT3 show both troubling similarities and subtle differences in terms of their distribution in the brain and their functional properties (Couroussé and Gautron 2015). The two OCTs belong to uptake 2 monoamine transport system, functioning as a compensatory clearance system of aminergic neurotransmitters in the brain. OCT3 is widely distributed in the brain, where it transports aminergic neurotransmitters, including dopamine, 5-HT, noradrenaline, and adrenaline, showing its contribution to monoamine homeostasis in the brain. Absence of Oct3 was associated with significant region-specific reductions in monoamine transmitters and histamine in mouse brain (Vialou et al. 2008). The most conspicuous difference between genotypes was a decrease in the level of dopamine in several regions, decreasing ranged from 46% in the substantia nigra/ventral tegmental area to 21% in the olfactory bulbs. The behavioral characterization of these OCT mutants reveals subtle behavioral alterations such as increased sensitivity to psychostimulants and increased levels of anxiety and stress (Vialou et al. 2008). Mouse experiment demonstrated that deficiency of Oct3 decreased anxiety in the elevated plus maze and open field (Wultsch et al. 2009). Clinical trial also confirmed association of OCT3 genetic variation and obsessive-compulsive disorder (Lazar et al. 2008).

Dysfunction of the serotonergic system is strongly linked to many psychiatric illnesses. 5-HT neurotransmission is tightly regulated by high-affinity uptake of released 5-HT by the 5-HT transporter, but brain OCT3 also mediates 5-HT transport. Oct3 expression was reported to be upregulated in the brains of mice with constitutively reduced 5-HT transporter expression. OCT blocker decynium-22 diminished 5-HT clearance from the brain, exerting antidepressant like effects in 5-HT transporter-deficient mice but not in wild-type mice (Baganz et al. 2008). Decynium-22 also enhanced antidepressant effects of the fluvoxamine in wild-type mice but not *Slc22a3*$^{-/-}$ mice (Horton et al. 2013). These data demonstrated an important role of OCT3 in the homeostatic regulation of serotonin neurotransmission and partly accounted for treatment resistance to antidepressants in some patients. Some antidepressants such as desipramine, sertraline, paroxetine, amitriptyline,

imipramine, and fluoxetine could inhibit OCT3 activity, indicating that the inhibitory effects on OCT3 activity also partly contributed to the overall therapeutic effects of antidepressants (Zhu et al. 2012).

Protein and mRNA of OCT3 are highly demonstrated in the dorsomedial hypothalamus. Consistent with the pharmacological profile of OCT3, corticosterone, 5-HT, estradiol, and decynium-22 dose-dependently inhibited histamine accumulation. Moreover, corticosterone and decynium-22 also inhibited efflux of [^{3}H]-MPP$^+$ from hypothalamic minces. These data indicate that corticosterone may acutely modulate physiological and behavioral responses to stressors by altering serotonergic neurotransmission via inhibiting OCT3 activity (Gasser et al. 2006; Hill and Gasser 2013). OCT3 protein is also located to nuclear membranes and endomembrane systems, including vesicular structures and mitochondria of both neuronal and glial perikarya, indicating that OCT3 not only contributes to the clearance of extracellular monoamines but also plays a role in their intracellular disposition and action (Gasser et al. 2017).

OCT2 is notably expressed in the limbic system, being implicated in anxiety and depression-related behaviors (Bacq et al. 2012). Genetic deletion of Oct2 in mice significantly reduced tissue concentrations of noradrenaline and 5-HT and in ex vivo uptake of both the two neurotransmitters in the presence of the dual 5-HT- noradrenaline transport blocker, venlafaxine. In the presence of venlafaxine, in vivo clearance of NE and 5-HT was diminished in the hippocampus of *Slc22a2*$^{-/-}$ mice, leading to alteration in postsynaptic neuronal activity. The mutant mice were insensitive to long-term venlafaxine treatment in a more realistic, corticosterone-induced, chronic depression model (Bacq et al. 2012). OCT2 was also detected in several stress-related circuits in the brain and along the hypothalamic-pituitary-adrenocortical axis. In mice, genetic deletion of Oct2 enhanced hormonal response to acute stress and impaired hypothalamic-pituitary-adrenocortical function without altering adrenal sensitivity to adrenocorticotropic hormone. Another report showed that the *Slc22a*$^{-/-}$ mice possessed more potent sensitivity to the action of unpredictable chronic mild stress on depression-related behaviors (Couroussé et al. 2015). These results infer that OCT2 mutations or blockade by certain therapeutic drugs could interfere with hypothalamic-pituitary-adrenocortical axis function, leading to stress-related disorders, and that OCT2 may be identified as a potential pharmacological target for mood disorders therapy (Bacq et al. 2012; Couroussé et al. 2015).

3.3.3.4 OCTs and Cancer

Accumulating evidences have demonstrated that some cancers may downregulate function and expression of OCTs/OCTNs, decreasing clinical outcome and therapeutic response to chemotherapeutic drugs. mRNA levels of OCT1 and OCT2 were significantly downregulated in hepatocellular carcinoma tissue (Heise et al. 2012) and cholangiocellular carcinoma tissues (Lautem et al. 2013) compared with nonneoplastic tumor surrounding tissue. The OCT1 downregulation was also

associated with advanced tumor stage and worse overall patient survival (Heise et al. 2012; Lautem et al. 2013; Grimm et al. 2016). Moreover, development of hepatocellular carcinoma and cholangiocarcinoma was accompanied by the appearance of aberrant OCT1 variants. The variants may dramatically affect distribution of sorafenib in tumor tissues (Herraez et al. 2013). Different from normal tissue, expression of OCT1 on surface membrane of carcinoma cells was weak or undetectable (Heise et al. 2012; Lautem et al. 2013), indicating the presence of nonfunctional OCT1 protein with abrogated transport function in tumor tissues. Importantly, the expression of OCT1 protein at the plasma membrane, rather than the overall OCT1 expression, was related with a favorable outcome in hepatocellular carcinoma patients treated with sorafenib (Geier et al. 2017).

Imatinib mesylate is considered as a highly effective therapy for chronic myeloid leukemia patients, but approximately 35% of patients fail to achieve optimal response. OCT1 mRNA expression was related to the maturation of the myeloid cells (Engler et al. 2011), increasing along with the differentiation of cells from blast forms to mature granulocytes. Both activity and mRNA expression of OCT1 were significantly lower in myeloid leukemia $CD34^+$ cells compared with mature $CD34^-$ cells (Engler et al. 2010; Stefanko et al. 2017), leading to low imatinib accumulation in primitive chronic myeloid leukemia cells. These findings seem to explain the fact that chronic myeloid leukemia progenitor cells are less sensitive to imatinib-induced apoptosis, being likely contributors to disease persistence. Several reports (Ben Hassine et al. 2017; da Cunha Vasconcelos et al. 2016; de Lima et al. 2014) have demonstrated that mRNA expression OCT1 is significantly downregulated in the leukocyte samples of the imatinib nonresponders. A clinical report demonstrated that chronic myeloid leukemia patients with high OCT1 activity achieved significantly greater molecular responses over 24 months of imatinib treatment than patients with low OCT1 activity (White et al. 2007). Patients with AA or GA genotypes of *rs628031* in OCT1 had a higher incidence of poor response to imatinib and a higher rate of kinase domain mutation discovery compared to the GG genotype (Koren-Michowitz et al. 2014). All these data indicate that OCT1expression or activity may be associated with a successful imatinib therapy in chronic myeloid leukemia patients.

Expression of OCT1 mRNA in acute myeloid leukemia patients was also significantly lower in comparison to the healthy controls (Stefanko et al. 2017). Patients with $CD34^+$ leukemia showed a visibly lower OCT1 mRNA level than $CD34^-$ patients, but patients with lower expression of OCT1 had higher chances to achieve complete remission and longer overall survival following treatment with daunorubicin and cytarabine arabinoside (Stefanko et al. 2017), which may in part attributed to positive correlation between the age of the patients and OCT1 mRNA level.

OCTN2 was also detected in breast cancer cell lines and tissue specimens. Estrogen receptor-positive cell lines or cancer tissues showed significantly higher OCTN2 expression compared with estrogen receptor-negative cell lines or cancer tissues. OCTN2 expression may be upregulated by estrogen, and OCTN2 is considered to be a critical regulator of carnitine homeostasis, lipid metabolism, and cell proliferation, indicating that OCTN2 may serve as a potential therapeutic target for

breast cancer (Wang et al. 2012). OCT6 is located in some cancer cell lines. In vitro studies (Oguri et al. 2016; Kunii et al. 2015) demonstrated that both cisplatin-resistant cells and oxaliplatin-resistant cells showed significantly lower expression of OCT6 than their parental cell lines, which were concomitant with lower concentration of platinum and cytotoxicity. The cisplatin-resistant cells, following overexpression of OCT6, exhibited high cytotoxicity of cisplatin and oxaliplatin, accompanied by increases in intracellular concentration of platinum, indicating that OCT6 is a mediator of platinum uptake in cancer cells and downregulation of OCT6 is possibly one of the mechanisms of resistance against cisplatin in cancer.

3.4 Organic Anion Transporters (OATs)

3.4.1 General Properties and Distribution of OATs

Organic anion transporters (OATs) and urate transporter 1(URAT1) are another family of multispecific transporters, encoded by the *SLC22/Slc22* gene superfamily. Ten OATs have been identified, which are divided into three branches (A, B, and C) according to their phylogenetic relationship. Branch A consists of OAT1 and OAT3; branch B is OAT6. Branch C includes D subgroup (consisting of OAT2 and OAT10), E subgroup (including OAT5, OAT7, OAT8, and OAT9), and F subgroup (including OAT4 and URAT1) (Burckhardt 2012). OAT proteins consist of 536–556 amino acids arranged in predicted 12 TMDs, whose N- and C-termini are both located intracellularly. A conspicuously large extracellular loop exists between TMD1 and TMD2, carrying several sites for N-linked glycosylation and conserved cysteine residues for the formation of disulfide cross bridges. A second large loop is located intracellularly between TMD6 and TMD7, carrying consensus sites for phosphorylation by several protein kinases (Burckhardt 2012).

OATs are located at almost all barrier epithelia of the body, endothelium, and other cells, demonstrating their roles in the regulated transcellular movement of numerous small organic anionic molecules across these epithelial barriers and between body fluid compartments. Although these transporters are capable of the bidirectional movement of substrates, most of the OATs are generally viewed as facilitating the movement of organic anions into the epithelial cells (influx transporters).

3.4.1.1 OAT1

Human OAT1 and animal Oat1 proteins are cloned by *SLC22A6* and *Slc22a6*, respectively. OAT1 is composed of 546 amino acids. Rat and mouse Oat1 have 551 and 546 amino acids, respectively. The highest expression of OAT1 mRNA is detected in the kidney, followed by the skeletal muscle, brain, and placenta (Roth et al. 2012). OAT1 protein is expressed at the basolateral membrane of proximal

tubules and in the plasma membrane of skeletal muscle cells. Human OAT1 protein is located along the whole proximal tubule segments 1–3, which is consistent with the role of OAT1 in taking up drugs from the blood into proximal tubule cells. Rat Oat1 protein is the highest in the segment 2. Expression of mouse Oat1 in segments 1 and 2 is higher than that in segment 3 (Burckhardt 2012). Proteins of mouse and rat Oat1 are expressed at the apical membrane of choroid plexus (Roth et al. 2012), although membrane localization of human OAT1 in the choroid plexus has not yet been identified.

3.4.1.2 OAT2

Human OAT2 and rodent Oat2 proteins are coded by *SLC22A7* and *Slc22a7*, respectively. Human OAT2 protein consists of 546 or 548 amino acids. The shorter splice variant of OAT2 (546 amino acids) lacking a serine and a glutamine in the large extracellular loop can be functionally expressed, whereas the 548-amino acid variant turned out to be nonfunctional (Cropp et al. 2008). Rat Oat2 and mouse Oat2 proteins have 535 and 540 amino acids, respectively. The highest levels of human OAT2 mRNA are demonstrated in the liver and kidneys, followed by the pancreas, small intestine, lung, brain, spinal cord, and heart. In male rats, Oat2 is predominantly expressed in the liver with smaller levels in the kidneys and other tissues, but in mature female rats, level of Oat2 mRNA in the kidneys is higher than that in the liver (Burckhardt 2012). In male mice, Oat2 mRNA is expressed only in the kidneys, whereas in female mice, it is expressed both in the liver and kidneys. Human OAT2 protein is localized to the basolateral membrane of proximal tubules. However, in rats and mice, Oat2 protein is expressed at the apical membrane in late S3 segments of proximal tubules, cortical thick ascending limbs of Henle's loop, and collecting ducts (Burckhardt 2012). Location of human OAT2 protein at membrane of human hepatocytes further needs investigation.

3.4.1.3 OAT3

Human OAT3 and rodent Oat3 proteins are cloned by *SLC22A8* and *Slc22a8*, respectively. The mammalian OAT3/Oat3 proteins have 536–542 amino acids. Human OAT3 mRNA has been detected in the kidneys, liver, brain, skeletal muscle, adrenal glands, and retina (Burckhardt 2012; Roth et al. 2012), of which the kidney shows the highest expression of OAT3 mRNA. Great gender differences in Oat3 expression exist in rodents. Levels of Oat3 mRNA in the liver of male rats and mice are higher than those in females. OAT3/Oat3 protein is localized to the basolateral membrane of human and rat renal proximal tubule, which is in line with the role of OAT3/Oat3 to take up organic anions and drugs from the blood into proximal tubule cells. OAT3 protein is also expressed in the basal membrane of brain capillary endothelial cells and in the choroid plexus, indicating that OAT3 may be involved in the clearance of anionic drugs from brain tissue and cerebrospinal fluid (Kikuchi

et al. 2014; Miyajima et al. 2011). In the retina, protein of OAT3 is expressed abluminal side of vascular endothelial cells where it appears to be involved in the efflux of organic anions and drugs from the vitreous humor to the blood (Hosoya et al. 2009).

3.4.1.4 OAT4

OAT4, consisting of 550 amino acids, is coded by *SLC22A11*; no orthologue was found in rodents. OAT4 mRNA is mainly expressed in the kidney and placenta. Adrenal tissue and the human adrenal cell line NCI-H295R also show expression of OAT4 mRNA (Roth et al. 2012). In the kidney, OAT4 is located at the luminal membrane of all segments of proximal tubules, whose function is either taking up organic anions from the primary urine or releasing organic anions from the cell into the tubule lumen. In the placenta, OAT4 is localized at the basal side of the syncytiotrophoblast where it takes up sulfated steroids from the fetal blood as precursors of placental estrogen synthesis during pregnancy (Burckhardt 2012; Roth et al. 2012). OAT4 is an antiporter exchanging organic anions against dicarboxylates. E3S and urate can be taken up from the primary urine into proximal tubule cells by exchange against intracellular α-ketoglutarate or, possibly, intracellular hydroxyl ions.

3.4.1.5 Other OATs

Human OAT5 is coded by *SLC22A10*, whose mRNA is only detected in the liver (Klein et al. 2010). Rat and mouse Oat5 is coded by *Slc22a19*; thus rodent Oat5 (*Slc22a19*) is not homologous to human OAT5. Rat and mouse Oat5 is restricted to the kidneys where it is expressed in the apical membrane of late proximal tubule cells (Burckhardt 2012). Human OAT7, a 553-amino acid protein, is coded by SLC22A9 and restricted to the liver where it is located in the sinusoidal membrane. Human OAT10, previously termed as "organic cation transporter like 3," is coded by *SLC22A13* (Burckhardt 2012; Nigam et al. 2015; Roth et al. 2012). OAT10 mRNA has been shown to have the highest expression in the kidney, followed by the brain, heart, small intestine, and colon. OAT10 protein is expressed in the apical membrane of proximal tubule cells which is probably responsible for uptake of the vitamin nicotinate from the primary urine into proximal tubule cells (Burckhardt 2012; Nigam et al. 2015; Roth et al. 2012). Human URAT1, previously named the renal-specific transporter, is coded by *SLC22A12*. Its mRNA is expressed in kidney and vascular smooth muscle cells. URAT1 protein is localized to the apical membrane of renal proximal tubules, where it mediates urate uptake from renal proximal tubule (Burckhardt 2012; Nigam et al. 2015; Roth et al. 2012).

OATs do not directly utilize ATP hydrolysis for energetization of substrate translocation. Most of OATs operate as anion exchangers. They couple the uptake of an organic anion into the cell to the release of another organic anion from the cell. That is,

they utilize intracellular-extracellular gradients of anions such as α-ketoglutarate, lactate, and nicotinate, to drive "uphill" uptake of organic anions against the inside negative membrane potential. Therefore, OAT-mediated transport is considered to be a tertiary active transport process. For transport mediated by OAT1, OAT2, and OAT3, the first step in this process is the counter transport of a substrate against its electrochemical gradient in exchange for the movement of an intracellular bicarboxylate (α-ketoglutarate for OAT1 and OAT3; succinate for OAT2) in the same direction as its electrochemical gradient (Burckhardt 2012; Kobayashi et al. 2005). After the gradient of dicarboxylate has been directed outward, it is then maintained by being metabolized and transported across the basolateral membrane into cells via the Na^+/dicarboxylate cotransporter (NADC3). The Na^+ gradient, which is directed inward and drives this process, is maintained by a transport step that requires energy, i.e., the extracellular transport of Na^+ is performed at the basolateral membrane by Na^+/K^+-ATPase (Fig. 3.7a). OAT4 is an antiporter exchanging organic anions against dicarboxylates. Substrates can be taken up from the primary urine into proximal tubule cells by exchange against intracellular α-ketoglutarate or hydroxyl ions. The gradients of dicarboxylates and hydroxyl ions are sustained by utilizing Na^+-coupled dicarboxylate cotransporter (NaDC1) and Na^+/H^+ exchanger (NHE3) in apical membrane to move the organic anion substrate into the cell and H^+ out of cell, respectively (Fig. 3.7b). OAT10 operates as an antiporter, exchanging extracellular urate or nicotinate against intracellular lactate, nicotinate, succinate, or glutathione (Fig. 3.7c). Human URAT1 is most probably the exchange of extracellular urate against intracellular lactate. Urate/lactate exchange leads to urate reabsorption in the proximal tubules. Lactate is absorbed back into the cell through the Na^+-monocarboxylate cotransporter (Fig. 3.7d).

3.4.2 Substrates and Inhibitors of OATs

OATs have been characterized as multispecific organic anion-dicarboxylate exchangers. OAT1 and OAT3 have been shown to transport organic anions against a negative membrane potential in exchange for the counter ion α-ketoglutarate. However, human OAT7 exhibits a unique exchange mechanism using short-chain fatty acids (such as butyrate) as counter ions for the transport of sulfate conjugates. OATs have broad substrate specificity and overlap each other. For example, OAT1 and OAT3 mediate p-aminohippurate transport, although p-aminohippurate affinity to OAT3 is slightly lower to OAT1 (Roth et al. 2012).

3.4.2.1 OAT1

OAT1 mediates transport of a vast number of endogenous and exogenous organic anions (Roth et al. 2012). Besides α-ketoglutarate, endogenous substrates of OAT1 include monocarboxylates (such as butyrate, lactate, propionate, and pyruvate),

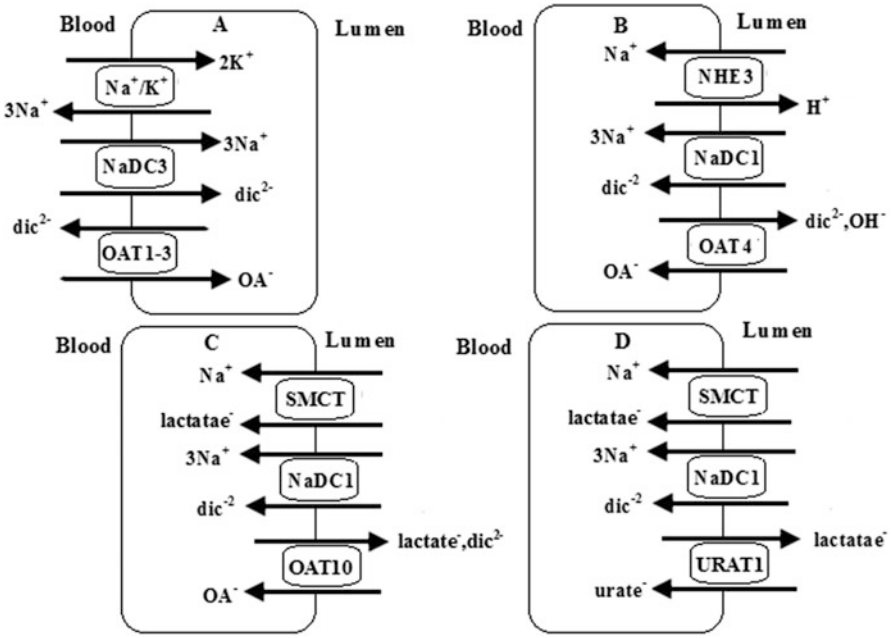

Fig. 3.7 Localization of OATs in human renal proximal tubule cell and the proposed transepithelial transport pathway for organic anions (OA^-). (**a**) Uptake of OA^- by OAT1-3 via exchanging against intracellular dicarboxylate (e.g., α-ketoglutarate or succinate); (**b**) uptake of OA^- by OAT4 via exchanging against intracellular dicarboxylate or hydroxyl ions; (**c**) uptake of nicotinate (or urate) by OAT10 via exchanging against intracellular dicarboxylate or lactate; and (**d**) uptake of urate by URAT1 via exchanging against intracellular lactate. Symbol: dic^{2-}, dicarboxylate; NaDC1, Na^+-dicarboxylate cotransporter in apical membrane; NaDC3, Na^+-dicarboxylate cotransporter in basolateral membrane; NHE3, Na^+/H^+ exchanger; Na^+/K^+, Na^+/K^+-ATPase; OH^-, hydroxyl ions; SMCT, Na^+-monocarboxylate cotransporter

short-chain fatty acids (such as hexanoate, heptanoate, and octanoate), prostaglandins (PGE2 and PGF2α), cyclic nucleotides (cAMP and cGMP), urate, folate, nicotinate, and a considerable number of acidic metabolites of neurotransmitter (such as vanillinemandelate, 3, 4-dihydroxyphenylacetate, homovanillate, and 5-hydroxyindoleacetate), tryptophan metabolism (quinolinate and kynurenate), and purine metabolites (such as xanthine and hypoxanthine) (Burckhardt 2012; Roth et al. 2012; Uwai et al. 2012). Numerous therapeutic drugs have been tested as possible substrates of human OAT1 including ACE inhibitors (captopril and quinapril), angiotensin II receptor blockers (candesartan, losartan, pratosartan, telmisartan, and valsartan), diuretics (bumetanide, chlorothiazide, cyclothiazide, ethacrynate, furosemide, furosemide, hydrochlorothiazide, torasemide, and trichloromethiazide), β-lactam antibiotics (ceftibuten and ceftizoxime), antiviral agents (acyclovir, adefovir, cidofovir, tenofovir, and zidovudine), H2 receptor antagonists (cimetidine and ranitidine), and NSAIDs (ibuprofen, indomethacin, ketoprofen, and salicylate) (Burckhardt 2012; Rizwan and Burckhardt 2007; Roth

et al. 2012). Most of them are also inhibitors of OAT1. Some compounds, not transported by human OAT1, may be potential OAT1 inhibitors, such as benzbromarone, diclofenac, diflunisal, flurbiprofen, mefenamate, mycophenolate, and probenecid. Endogenous compounds such as kynurenic acid and α-ketoglutarate are also strong inhibitors of human OAT1 (Ingraham et al. 2014; Uwai et al. 2012). Interestingly, physiologic concentration of α-ketoglutarate reduces the inhibitory potency of OAT1 inhibitors in substrate- and inhibitor-dependent manner. For example, p-aminohippurate was used for the substrate; α-ketoglutarate significantly increased the IC_{50} values of probenecid, indomethacin, naproxen, ibuprofen, bumetanide, cephalothin, and ceftriaxone; but this increase was not seen for furosemide, valsartan, and telmisartan. However, α-ketoglutarate significantly increased the IC_{50} values of furosemide and valsartan when cidofovir was used for the substrate (Ingraham et al. 2014).

3.4.2.2 OAT2

Human OAT2 functions as an antiporter, exchanging intracellular succinate or fumarate, not glutarate, against extracellular substrates. Endogenous compounds transported by human OAT2 include L-ascorbate, glutamate, glutarate, DHEAS, E3S, nucleobases, nucleosides and nucleotides, orotate, prostaglandins (PGE2 and PGF2α), and urate (Burckhardt 2012; Fork et al. 2011; Kobayashi et al. 2005; Sato et al. 2010). 2-Deoxyguanosine and cGMP are good substrates for human OAT2, but not rat Oat2 (Fork et al. 2011). OAT2, OCT2, MATE1, and MATE2K also mediate transport of creatinine, but the initial rate of OAT2-mediated creatinine transport was reported to be approximately 11-, 80-, and 80-fold higher than OCT2, MATE1, and MATE2K, respectively, indicating that the OAT2-mediated creatinine transport is far more efficient than OCT2, MATE1, and MATE2K (Shen et al. 2015).

Types of intracellular organic anions exchanging against extracellular substrates by OAT2 are dependent on substrates (Fork et al. 2011; Henjakovic et al. 2015; Kobayashi et al. 2005; Pfennig et al. 2013). When cGMP was used as substrate, OAT2-mediated uptake of cGMP was inhibited by PGE2 and PGF2α, but not short-chain monocarboxylates nor dicarboxylates. OAT2-mediated cGMP uptake is also independent of pH, Cl^-, or membrane potential. In addition, OAT2 transports urate and glutamate, but no cGMP/glutamate exchange occurs. These results indicate that OAT2-mediated cGMP uptake does not occur via exchange with monocarboxylates, dicarboxylates, and hydroxyl ions (Henjakovic et al. 2015). When orotic acid was used as substrate, the force driving uptake of orotic acid by OAT2 was identified as glutamate antiport (Fork et al. 2011). OAT2 itself physiologically functions as a glutamate efflux transporter in hepatocytes (Fork et al. 2011). The glutamate efflux by OAT2 may be stimulated by benzoic acid and 2-oxo-leucine, not 2-oxo-valine (Pfennig et al. 2013). When E3S was served as substrate, both fumarate and succinate stimulated OAT2-mediated uptake of E3S (Kobayashi et al. 2005).

Several therapeutic drugs including antivirals (such as acyclovir, ganciclovir, and penciclovir), antineoplastic drugs (such as bendamustine, 6-fluorouracil,

irinotecan, methotrexate, and paclitaxel), anti-gout agents (allopurinol), and diuretics (bumetanide) have been identified as OAT2 substrates (Burckhardt 2012; Cheng et al. 2012; Kobayashi et al. 2005; Marada et al. 2015). Several compounds including NSAIDs (such as diclofenac, ibuprofen, indomethacin, ketoprofen, mefenamate, naproxen, piroxicam, and sulindac) and diuretics (such as bumetanide, hydrochlorothiazide, and trichloromethiazide) have been identified as inhibitors of OAT2, but some of them are not translocated by human OAT2 (Burckhardt 2012). Importantly, indomethacin shows strong inhibition on human OAT2 activity, which may be used for selective inhibitor of OAT2 (Henjakovic et al. 2015). BSP is a strong inhibitor for mouse Oat2-mediated uptake, but this compound stimulates human OAT2-mediated uptake of tetracycline, which indicates that the substrate selectivity of human OAT2 is different from that of mouse Oat2 (Kobayashi et al. 2005).

3.4.2.3 OAT3

OAT3 operates as an antiporter, exchanging intracellular α-ketoglutarate against extracellular organic anions and drugs. OAT1 and OAT3 share the specificity for dicarboxylates, but, in general, OAT3 has a lower affinity toward these compounds. E3S is the preferred test substrate for OAT3. Endogenous substrates transported by OAT3 include the second messengers (cAMP and cGMP), the bile salts (cholate and taurocholate), the hormones (cortisol, DHEAS, and E3S), the prostaglandins (PGE2 and PGF2α), urate, and kynurenate (Burckhardt 2012; Uwai et al. 2012). Urinary excretion of 6β-hydroxycortisol, a metabolite of cortisol, is mainly mediated by OAT3 and may be served as an endogenous probe to investigate the perpetrators of the pharmacokinetic drug interactions involving OAT3 in humans (Imamura et al. 2014). Human OAT3 also transports some drugs including angiotensin II receptor blockers (olmesartan), diuretics (bumetanide, ethacrynate, and furosemide), and β-lactam antibiotics (cefaclor, cefazolin, cefdinir, cefoselis, cefotiam, ceftizoxime, and cephaloridine) (Burckhardt 2012). The uptake of cephaloridine by rat Oat3 was considered to contribute to cephaloridine nephrotoxicity (Jung et al. 2002). Methotrexate is transported by human OAT3 and OAT1, but the OAT3 affinity to methotrexate is much higher than that of OAT1, suggesting that OAT3 is mainly responsible for renal methotrexate excretion (Burckhardt 2012).

A series of compounds have been identified as inhibitors of OAT3 including endogenous compounds (kynurenine and kynurenic acid) and therapeutic drugs (Burckhardt 2012; Uwai et al. 2012). Angiotensin II receptor blockers (candesartan, losartan, olmesartan, pratosartan, telmisartan, and valsartan), diuretics (bumetanide, ethacrynate, and furosemide), and NSAIDs (diclofenac, ibuprofen, indomethacin, ketoprofen, loxoprofen, mefenamate, naproxen, phenylbutazone, and piroxicam) show potent affinities to human OAT3 whose IC_{50} values are less than 10 μM, inferring that these drugs can give rise to drug-drug interactions (Burckhardt 2012).

3.4.3 Pharmacological and Physiological Function of OATs

3.4.3.1 Impact of OATs on Kidney Drug Levels and Action

OAT1 and OAT3 are mainly located at the basolateral membrane of renal proximal tubule cells, where they mediate excretion of their substrates (organic anions). In the kidney, these organic anions are secreted in renal proximal tubules in two steps. First, they are transported from the blood to proximal tubular cells across basolateral membranes. These processes are usually mediated by influx transporters such as OAT1 and OAT3. Then organic anions were effluxed into urine across apical membranes of proximal tubules via efflux transporters. Moreover, some of them in proximal tubules may reabsorbed into proximal tubular cells via influx transporters such as OAT4, OAT10, and URAT1.

Creatinine is substrate of OATs especially OAT2 (Lepist et al. 2014; Shen et al. 2015). Although other transporter such as OCT2, MATE1, and MATE2K also mediate creatinine transport, OAT2 has demonstrated the most efficient transport of creatinine (Lepist et al. 2014; Shen et al. 2015). Some drugs including angiotensin II receptor blockers, diuretics, and NSAIDs are potential inhibitors of OATs, which may at least partly explain some clinic findings. For example, NSAIDs were reported to rise levels of creatinine in patients of rheumatoid arthritis, although the rise of serum creatinine was reversible in most patients (Pathan et al. 2003). A meta-analysis (Lee et al. 1999) also demonstrated that NSAIDs reduced creatinine clearance and increased serum creatinine without altering urine volume. Similarly, treatment of candesartan was reported to be associated with increase in serum creatinine and decrease in estimated glomerular filtration rate (Damman et al. 2014, 2016).

Urate is also endogenous substrate of OATs, although other transporters also mediate urate excretion in urine. Some drugs may affect urate excretion in urine via inhibiting OAT function, rising crisis of gout. Hyperuricemia is a well-known adverse effect of diuretic treatment which are thought to have direct actions on urate transporters in renal proximal. Common diuretics, such as loop diuretics (furosemide and bumetanide) and thiazides, also reduce urinary uric acid excretion and increase the serum uric acid level (Russel et al. 2002). A clinic report (McAdams DeMarco et al. 2012) on 5789 participants with hypertension showed that use of diuretics was highly associated with incident gout and higher serum urate levels. Similarly, low dose of hydrochlorothiazide coadministrated with telmisartan or candesartan significantly increased the serum urate level and decreased fractional excretion of urate in urine (Hamada et al. 2010). But these alterations did not occur in patient treated with hydrochlorothiazide with losartan. On the contrast, losartan reduced serum uric acid levels (Fan et al. 2015; Matsumura et al. 2015). These results may be explained by following facts that angiotensin II receptor blockers (telmisartan, candesartan, and losartan) all inhibit OAT1- and OAT3-mediated urate uptake (Sato et al. 2008), but only losartan shows potential inhibition on URAT1 (Iwanaga et al. 2007), inhibiting reabsorption of urate from proximal tubules.

Chronic kidney disease is characterized by the accumulation of uremic toxins in plasma due to deterioration of renal function. These uremic toxins are often substrates or inhibitors of OATs (Hsueh et al. 2016; Masereeuw et al. 2014). These uremic toxins enhance their accumulation in plasma via inhibiting their excretion in urine. On the other hand, the increased uremic toxins may affect function and expression of renal OATs. For example, acute kidney injury rats induced by ischemia, ureteral obstruction, or the administration of $HgCl_2$ significantly decreased expression of renal Oat1 and Oat2, accompanied by increased urea plasma levels (Brandoni and Torres 2015). In mice, Oat1 deficiency was associated with high plasma concentrations and/or lower urinary concentrations of endogenous organic anions (Eraly et al. 2006). These results demonstrate roles of OATs in accumulation of uremic toxins under chronic kidney disease. Moreover, these uremic toxins inhibit OAT1 and OAT3 function progressively, causing severe problems with the excretion of clinically used drugs including antibiotics, methotrexate, and antivirals in patients with chronic kidney disease (Hsueh et al. 2016), altering drug efficacy and toxicity.

OATs are also implicated in in the progression of chronic renal failure via transporting uremic toxins into cells. For example, levels of indoxyl sulfate, a metabolite from food-derived indole, was significantly elevated in plasma of patients with chronic renal failure. This compound is considered to be a factor involved in the progression of chronic renal failure. Indoxyl sulfate is transported into cells via OATs, accumulates there, and causes free radical production and finally induces nephrotoxicity (Enomoto and Niwa 2007).

OATs show broad substrate specificity, inferring occurrence of drug-drug interactions when drugs are mainly eliminated by OAT1 or OAT3 in the kidneys and coadministrated. For example, adefovir and benzylpenicillin are specifically transported by OAT1 and OAT3, respectively. In human, ratios of the free renal clearance to glomerular filtration for adefovir and benzylpenicillin are greater than 1, indicating that the two drugs undergo renal tubular secretion. Coadministration of OAT1inhibitor p-aminohippurate or OAT3 inhibitor probenecid significantly reduced the renal clearance of adefovir. In line, coadministration probenecid (1500 mg) also decreased renal clearance of benzylpenicillin (Maeda et al. 2014). In rats, coadministration of benzylpenicillin markedly increased plasma concentration of acyclovir and decreased accumulative renal excretion and renal clearance of acyclovir, which may be due to inhibition of Oat1 and Oat3 (Ye et al. 2013).

Renal OAT1 and OAT3 may be implicated in drug-induced nephrotoxicity. OAT1, OAT3, and OAT4 contribute to nephrotoxicity of cephaloridine and antiviral drugs via taking up them from the blood into the proximal tubular cells (Rizwan and Burckhardt 2007; Takeda et al. 2002). Strategies to prevent their nephrotoxicity involve the use of inhibitors of OATs such as probenecid and betamipron (Rizwan and Burckhardt 2007; Takeda et al. 2001). Crystal nephropathy is a well-known adverse side effect of acyclovir with its low solubility in urine, in particular when applied intravenously at high doses (Rizwan and Burckhardt 2007). Acyclovir elimination occurs to an average of ~65% as unchanged drug via the kidneys by glomerular filtration as well as tubular secretion. The tubular secretion is mainly

mediated by OAT1 and substantially inhibited by coadministration with probenecid or cimetidine (Hagos and Wolff 2010). Cidofovir, another antiviral, results in nephrotoxicity due to high exposure to proximal tubular epithelial cells (Nieskens et al. 2016). In monkey, coadministration of probenecid might completely prevent cidofovir-mediated nephrotoxicity, accompanied by significant decrease in renal clearance (Lacy et al. 1998). In vitro study showed that overexpression of OAT1 was associated with increased cytotoxicity of adefovir or cidofovir (Ho et al. 2000) and that NSAIDs (such as diflunisal, ketoprofen, flurbiprofen, indomethacin, naproxen, and ibuprofen) reduced adefovir cytotoxicity via inhibiting OAT1 activity (Mulato et al. 2000). These findings indicate that the use of OAT inhibitors (such as NSAIDs and probenecid) can reduce renal excretion of antiviral drugs and their nephrotoxicity (Mulato et al. 2000; Uwai et al. 2007). Methotrexate-induced nephrotoxicity is also associated with renal OAT1. A report showed that recombinant EGFR antibody cetuximab may attenuate methotrexate-induced cytotoxicity, which may be partly attributed to downregulation of OAT1 expression (Caetano-Pinto et al. 2017). The primary site of mercury-induced injury is the kidney due to uptake of the reactive Hg^{2+}-conjugated organic anions in the proximal tubule which may be attributed to Oat1 expression. In mice, *Oat1* deficiency abolished renal injury by $HgCl_2$ (Torres et al. 2011). Similarly, nephrotoxicity of aristolochic acid I is partly attributed to expression of renal Oat1 and Oat3 (Xue et al. 2011).

Proximal tubular epithelial cells are targeted cells of diuretics. Some diuretics are also substrates of OATs, indicating that alterations in renal OAT activities affect diuretic response. Oat1 or Oat3 deficiency was reported to impair natriuretic response to both furosemide and bendroflumethiazide in mice, leading to a rightward shift in the natriuresis dose-response curve and increases in ED_{50} (Vallon et al. 2008a; Eraly et al. 2006). These alterations were consistent with decreased renal excretion.

3.4.3.2 Impact of OATs on Brain Drug Levels and Action

Expression of OATs has been demonstrated in the brain and choroid plexus, whose functions are to efflux toxins, drugs, and metabolites from the cerebrospinal fluid (CSF) and central nervous system (CNS), indicating roles of brain OATs in determining brain levels of drugs, toxins, and metabolites and affecting their function and toxicity in CNS. OAT1 and OAT3 are expressed in apical side of choroid plexus, forming blood-cerebrospinal fluid barrier (BCSFB). A report showed that antivirals (acyclovir, lamivudine, tenofovir, and zidovudine) manifested significant interaction with both OAT1 and OAT3, inferring that brain OAT1 and OAT3 have important implications for antiretroviral penetration into or retention within the CNS (Nagle et al. 2013). Bumetanide also exerts effects on brain disorders, including autism, neonatal seizures, and epilepsy via inhibiting the neuronal Na-K-Cl cotransporter isoform. However, poor brain penetration of bumetanide limits its clinical use for treatment of brain disorders. Bumetanide is OAT3 (Donovan et al. 2016), and poor brain penetration of bumetanide may be attributed to expression of brain OAT3.

Coadministration of OAT3 inhibitor probenecid was reported to increase brain levels of bumetanide (Donovan et al. 2014; Römermann et al. 2017; Töllner et al. 2015), indicating that bumetanide/probenecid combination may hold therapeutic potential.

Acid metabolites of some neurotransmitters and amino acids, such as quinolinic acid, kynurenic acid, 5-hydroxyindoleacetic acid, and homovanillic acid, are substrates of human OAT1 and OAT3 (Uwai et al. 2012; Burckhardt 2012). Brain OAT3 and OAT1 transport these compounds from brain to blood across BBB or BCSFB to protect their accumulation in the brain. Several reports have demonstrated that these metabolites modulate several neurotransmitter systems and that impairment of their balance leads to a variety of diseases of the brain, such as neurotoxicity, Huntington's disease, seizure, Alzheimer's disease, and Parkinson's disease (Lim et al. 2017; Nilsson et al. 2005; Plitman et al. 2017; Schwarcz and Pellicciari 2002). Roles of OAT1 in the process of Alzheimer's disease were also confirmed using *Slc22a6*-deficient tg2576 mice. The *Slc22a6*$^{-/-}$ tg2576 mice showed impaired learning and memory behavior as well as reduced long-term potentiation level and spontaneous excitatory postsynaptic currents frequency (Wu et al. 2015b).

Kynurenic acid is known to block N-methyl D-aspartate (NMDA) receptor and α7 nicotinic acetylcholine receptor in the brain (Erhardt et al. 2009). Several reports have demonstrated the increased concentration of kynurenic acid in the brain of schizophrenic patients, suggesting that kynurenic acid is one of the key molecules in schizophrenia (Kozak et al. 2014; Linderholm et al. 2012; Nilsson et al. 2005; Plitman et al. 2017) and that brain OAT1 and OAT3 are implicated in diseases of the brain via removing the compound from the brain. In addition, kynurenic acid inhibits the uptake of some drugs (such as methotrexate and penciclovir) by OAT1 and OAT3, leading to drug-kynurenic acid interaction (Uwai et al. 2012). Brain OTA3 also effluxes E3S and DHEAS across BBB. Elimination of DHEAS from the brain after microinjection into the cerebral cortex of mice was reported to be completely inhibited by probenecid, benzylpenicillin, and E3S. In consistence, efflux of E3S or DHEAS from the brain was significantly delayed in *Slc22a8*-deficient mice (Miyajima et al. 2011).

Concentrations of PGE2 and PGD2 in brain interstitial fluid and cerebrospinal fluid are maintained at appropriate levels for normal brain function, which is mainly attributed to efflux transporters (OAT3, MRP4, and PG transporter) on BBB and BCSFB (Tachikawa et al. 2012a, b, 2014). PGE2 and PGD2 are substrates of OAT3. Data from isolated choroid plexus of rats confirmed that Oat3 mediated uptakes of PGD2 and PGE2, which was inhibited by OAT3 inhibitors/substrates (such as benzylpenicillin, probenecid, indomethacin, and diclofenac) (Tachikawa et al. 2012a, b). In vivo study also demonstrated that simultaneous injection of unlabeled PGE2 or β-lactam antibiotics (such as cefazolin and ceftriaxone) significantly inhibited PGE2 elimination clearance from the CSF (Tachikawa et al. 2012a; Akanuma et al. 2010), in turn affecting CNS function via a resulting increase in the brain level of PGE_2. Interestingly, PGE2 elimination across the BBB was attenuated in an LPS-induced mouse model of inflammation, which was consistent with decrease in expression of Oat3 protein. Moreover, peripheral administration of cefmetazole further inhibited PGE2 elimination from brain in LPS-treated mice

(Akanuma et al. 2011). PGE2 and PGD2 are well known to be involved in multiple brain pathophysiological processes, including modulation of synaptic plasticity, neuroinflammation, and sleep promotion. These findings indicate contribution of the brain OAT3 to pathophysiology and pharmacology of PGE2 and PGD2 (Tachikawa et al. 2012a, b, 2014).

Chronic kidney disease is associated with the accumulation of uremic toxins in plasma and uremic encephalopathy. These patients often have a higher risk of developing cognitive disorders and dementia (Bugnicourt et al. 2013). Although the pathophysiology of uremic encephalopathy remains poorly understood, accumulation of uremic toxins, hormonal disturbances, altered intermediate metabolism, as well as an imbalance of excitatory/inhibitory neurotransmitters contribute to the epileptic and cognitive symptoms accompanied by uremic encephalopathy. These uremic toxins are substrates or inhibitors of OAT1 and OAT3 (Deguchi et al. 2006; Hsueh et al. 2016; Masereeuw et al. 2014; Ohtsuki et al. 2002). Under physiological conditions, the concentrations of these uremic toxins (such as indoxyl sulfate, hippuric acid, 3-carboxy-4-methyl-5-propyl-2-furanpropionate, hippurate, and indoleacetate) in brain tissues are several times lower than those in serum. This disparity in distribution of compounds may be due to brain-to-blood transport of these uremic toxins mediated by OAT3 and OAT1 at the BBB, although other transporters such as OATP2 also mediate transport of 3-carboxy-4-methyl-5-propyl-2-furanpropionate (Deguchi et al. 2006). Chronic kidney disease was reported to significantly increase levels of hippurate, indoleacetate, and 3-carboxy-4-methyl-5-propyl-2-furanpropionate in serum, CSF, and brain tissue of patients (Hosoya and Tachikawa 2011). Accumulation of these uremic toxins, particularly hippuric acid, led to accumulation of neurotransmitter metabolites in brain of uremic patients via directly inhibiting OAT3 transport activity at the BBB, in turn, affecting neurotransmitter metabolic pathways. For example, the clinic report showed that patients with uremic encephalopathy had up to 4.5 times higher 5-hydroxyindoleacetic acid and homovanillic concentrations in the CSF (Hosoya and Tachikawa 2011). Intermediate metabolites (neurotoxins) of catecholamines, such as 4-dihydroxyphenylacetaldehyde and 3,4-dihydroxyphenylglycolaldehyde, were also reported to be linked to Alzheimer's and Parkinson's diseases (Hosoya and Tachikawa 2011). Moreover, patients with uremic encephalopathy was associated with high levels of indoxyl sulfate (Yeh et al. 2016). These findings indicate that renal failure results in accumulation of uremic toxins in the blood and that these increased uremic toxins inhibit efflux transport of organic anion from the brain, leading to interaction with neurotransmitter metabolites and uremic toxins, and uremic encephalopathy occurs (Hosoya and Tachikawa 2011).

3.4.3.3 Role of OATs in Cancer Development and Cancer Treatment

OATs mediate transport of some antineoplastic drugs including irinotecan, paclitaxel, chlorambucil, 5-fluorouracil, and bendamustine (Hagos et al. 2015; Kobayashi et al. 2005; Marada et al. 2015; Nishino et al. 2013; Tashiro et al. 2014), inferring

roles of OATs in cancer development and cancer treatment. For example, uracil/ftorafur is basic treatment for advanced colorectal cancer. A report from biopsy specimens of 45 patients showed that high expression levels of OAT2 were significantly correlated with a good response to uracil-/ftorafur-based chemotherapy (Nishino et al. 2013). Metastatic colorectal cancer is commonly treated with 5-fluorouracil/leucovorin/oxaliplatin. OAT2 and OCT2 are critical determinants in uptake of 5-fluorouracil and oxaliplatin, respectively. In 90 patients treated with 5-fluorouracil/leucovorin/oxaliplatin, high expressions of OAT2 and OCT2 were detected in 36% and 60% of the tumor patients, respectively. Furthermore, high expression of OAT2 was significantly correlated with good objective tumor response whereas high expression of OCT2 was associated with long progression-free survival. In addition, patients with both high expression of OAT2 and OCT2 showed the best treatment outcomes with significantly higher frequency than patients with other expression patterns (Tashiro et al. 2014). Chronic lymphatic leukemia is often treated with chlorambucil or bendamustine. A report showed that both lymphoma cell lines and primary chronic lymphatic leukemia possessed high expression of OAT3 and that OAT3-mediated accumulation of bendamustine was associated with reduced cell proliferation and an increased rate of apoptosis (Hagos et al. 2015). In vitro study showed that incubation of bendamustine with HEK 293 cell expressing OAT2 increased the caspase-3 activity, and this increase by bendamustine was inhibited by probenecid (Marada et al. 2015). These data indicate that the high efficacy of bendamustine in treating chronic lymphatic leukemia may be partly contributed to the expression of OAT3 in lymphoma cells (Hagos et al. 2015). It was reported that alpha-fetoprotein-producing gastric cancer had higher expression levels of both OAT2 and human equilibrative nucleoside transporter 1 (ENT1) compared with conventional gastric cancer. ENT1 and OAT2 are considered to be crucial for the uptake of gemcitabine and 5-fluorouracil, respectively, suggesting that patients with alpha-fetoprotein-producing gastric cancer could potentially benefit from gemcitabine/fluoropyrimidine combination chemotherapy and that the increased expression of ENT1 and OAT2 may also be associated with the progression of gastric cancer (Shimakata et al. 2016). Antifolates methotrexate and pemetrexed have been used in the treatment of primary and secondary tumors of the CNS. OAT3 mediates efflux of the two compounds from the brain, which was inhibited by probenecid and benzylpenicillin (Li et al. 2013a), inferring that patients may benefit from coadministration of antifolates and OAT3 inhibitors.

3.4.3.4 Roles of OATs in Remote Sensing and Signalling

OAT transporters (and ABC families as well as OCTs) mediate the movement of small endogenous molecules such as key metabolites, signalling molecules, vitamins, antioxidants, odorants, hormones, and conjugated steroids between tissues, organs, and even organisms. The distribution of the OATs in diverse organs, the presence of the same OAT isoform in different tissues, and their overlapping substrate specificities suggest that the OATs may participate in a broader communication network between

organs/tissues via transporting signalling molecules and metabolites, which leads to a "remote sensing and signalling" hypothesis (Fig. 3.8), although the argument is broadly applicable to other transporters such as ABC family and OCTs (Ahn and Nigam 2009; Wu et al. 2011; Nigam et al. 2015). According to the hypothesis, these transporters located in the barrier epithelia of certain organs may take up substrates excreted by transporters in other organs and mediate potential remote signalling and communication between organs (Ahn and Nigam 2009; Wu et al. 2011; Nigam et al. 2015). The OAT1 and OAT3, predominantly expressed in the kidney, secrete corresponding substrates into urine. Some compounds are also secreted into feces via corresponding transporters. OAT6 expressed in the olfactory epithelium, sharing 40 and 60% sequence homology with OAT1 and OAT3, respectively, possesses a significant affinity for small volatile compounds identified as odorant molecules in urine and feces. These odorant molecules in feces and urine may be taken up by the olfactory mucosa though an OAT6-mediated mechanism, which constitutes a signalling mechanism between organisms (Ahn and Nigam 2009; Wu et al. 2011; Nigam et al. 2015). OAT6 may modulate signalling from the olfactory epithelium to distant organs such as the brain (Ahn and Nigam 2009).

The OATs play most important roles in a broader remote-sensing system. In mice, it was reported that Oat1 deficiency significantly increased plasma concentrations and/or decreased urinary concentrations of several endogenous organic anions (such as 3-hydroxyisobutyrate, 3-hydroxybutyrate, 3-hydroxypropionate, benzoate, and N-acetylaspartate) compared with wild-type mice (Eraly et al. 2006). OAT1 may regulate several pathways involving essential metabolites (e.g., intermediates of tricarboxylic acid cycle, tryptophan metabolites derived from the gut microbiome), key signalling molecules (e.g., prostaglandins, polyamines, cyclic nucleotides), molecules with antioxidant activity (e.g., ascorbic acid, urate), hormones (e.g., thyroxine), vitamins, and cofactors (e.g., pantothenic acid), constructing "OAT1-centered metabolic network" (Liu et al. 2016; Ahn et al. 2011). These molecules in the network are considered to be central to classical metabolism and signalling pathways, as well as organ and systemic physiology, which are implicated in pathophysiological processes like hyperuricemia, metabolic syndrome, diabetes, and chronic kidney disease. Some metabolites transported by OAT1 are potential classical uremic toxins (e.g., indoxyl sulfate, kynurenate, polyamines, and uric acid). These uremic toxins accumulate in plasma of chronic kidney disease patients due to deterioration of renal function and inhibition of renal OATs (Hsueh et al. 2016). The increased uremic toxins cause interaction between neurotransmitter metabolites and uremic toxins via inhibiting efflux transport of these organic anions from the brain, leading to occurrence of uremic encephalopathy (Hosoya and Tachikawa 2011).

OAT3 also contributes to the metabolic network. It was reported that Oat3 deficiency led to significant alterations in some cellular metabolic pathways (such as tricarboxylic acid cycle, nucleotide, and amino acid metabolism), the expression of genes encoding drug metabolic enzymes and drug transporter, pathways involved in the regulation of secondary metabolites including endogenous signalling molecules (and dietary plant derivatives), and pathways involved in the handling of dietary flavonoids in mice (Wu et al. 2013). These alterations, in turn, may affect function

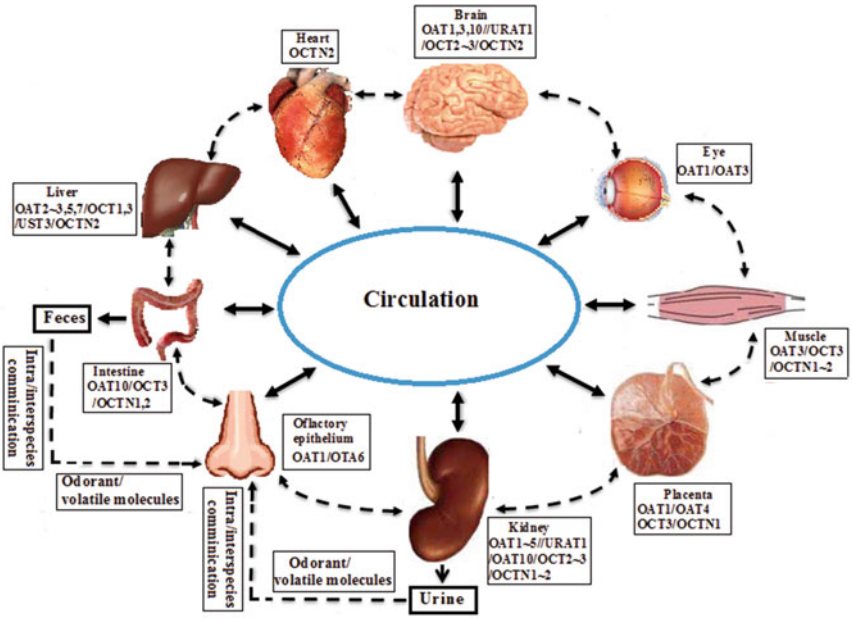

Fig. 3.8 Communication between organs/organisms mediated by SLC22 family members. Organic anion transporters (OATs), organic cation transporters (OCTs), organic carnitine transporters (OCTNs), URAT1, and unknown substrate transporters (USTs) located in the barrier epithelia of certain organs may take up substrates excreted by transporters in other organs and mediate potential remote signalling and communication between organs. Excreted odorant molecules in urine or feces may be taken up by the OAT6 or OAT1 in the olfactory mucosa, carried out intra-/interspecies communication

of multiple tissues in different mechanisms. In accordance with this, *Slc22a8*$^{-/-}$ mice were report to manifest a 10–15% lower blood pressure than wild-type mice, suggesting possible involvement of Oat3 in blood pressure regulation (Vallon et al. 2008b). Metabolomic analyses demonstrated that in mice, *Slc22a8* knockout significantly increased plasma levels of several potential Oat3 substrates (such as thymidine). These compounds may serve as endogenous blood pressure regulators. Consistently, intravenous administration of thymidine to mice resulted in a 10–15% reduction in blood pressure. OAT3 inhibitors eosin-Y or probenecid also resulted in reduction of blood pressure when administered to mice. The remote sensing circuit may partly explain changes in blood pressure due to changes in renal OAT3 expression or function. Elevated blood pressure sensed by the kidney may lead to decreased renal OAT3 expression or function and therefore reduced renal excretion of vasodilators. The vasodilators would accumulate in the body and subsequently be taken up by Oat3 expressed in the vascular smooth muscle, resulting in lowered blood pressure. This potential involvement of Oat3 in blood pressure regulation supports a central role of the Oats in responding to changes in the internal and external environment and thereby maintaining whole-body homeostasis (Ahn and Nigam 2009).

3.5 Multidrug and Toxin Extrusion (MATEs)

3.5.1 General Properties and Tissue Distribution of MATEs

Multidrug and toxin extrusion (MATE for human and Mate for other species, respectively) proteins, coded by SLC47A/Slc47a family, are H^+/organic cation antiporters directly transporting organic cations out of cells (Aleksunes et al. 2008). The first human orthologue, MATE1, was identified in 2005 (Otsuka et al. 2005). Shortly after, MATE2, MATE2-K, and MATE2-B were identified. Human MATE1 and MATE2 proteins are coded by the *SLC47A1* and *SLC47A2*, respectively. MATE2-K and MATE2-B are two alternatively spliced variants of MATE2. Compared to MATE2 cDNA, the MATE2-K cDNA lacks 108 base pairs in exon 7, and the MATE2-B cDNA contains an insertion of 46 base pairs in exon 7; they code a 566-amino acid protein and a 220-amino protein, respectively (Motohashi and Inui 2013; Staud et al. 2013).

Human MATE2 and MATE2-K share 94% amino acid similarity, and the sequence identity with MATE1 is 48% and 51%, respectively (Staud et al. 2013). Although human MATE1 and rodent Mate1 show high similarity, rodent Mate2 shows low sequence identity (38.1%) with human MATE2 variants (MATE2, MATE2-K, and MATE2-B); therefore rodent Mate2 could be classified into a third family based on sequence similarity. In addition, tissue distributions of MATE2 and Mate2 mRNA show significant difference between human and mouse. Mouse Mate2 mRNA was detected specifically in the testes (Lickteig et al. 2008). Rodent orthologues of human MATE2-K have not been identified. However, the rabbit orthologues have 75% (Mate1) and 74% (Mate2-K) amino acid identity to their human counterparts (Motohashi and Inui 2013; Yonezawa and Inui 2011; Staud et al. 2013; Terada and Inui 2008). Thus, care is needed when discussing the nomenclature and classification of MATE2/Mate2.

MATEs show notable interspecies differences in their expression. In human, MATE1 is highly expressed in the luminal membranes of the renal tubules and the bile canaliculi although it is also expressed in other tissues such as the adrenal gland, skeletal muscle, testis, and first trimester placenta. Human MATE2-K exhibits a kidney-specific expression. MATE1 and MATE2-K mRNA are detected at similar levels in the kidney, and these proteins are similarly localized in the luminal membrane of proximal tubules, inferring roles of the two transporters in the renal tubular secretion of cationic drugs. MATE2-B mRNA was found in all human tissues except the kidney, but its physiological roles are unclear (Motohashi and Inui 2013; Staud et al. 2013; Yonezawa and Inui 2011). In rats, Mate1 mRNA is expressed abundantly in the kidney and placenta, slightly in the spleen but not in the liver. In mice, Mate1 is highly expressed in the kidney, liver, heart, and several tissues. The tissue distribution of Mate1 in mice is generally consistent with that in human. However, Mate2-K is not expressed in mice, inferring that Mate1 knockout mice could represent a model of MATE1 and MATE2-K deficiency in humans (Yonezawa and Inui 2011).

Mammalian MATEs have 13 TMDs with their C-terminus on the extracellular face of the membranes. Several conversed amino acid residues such as histidine, cysteine, and glutamate acid are required for function of MATEs. The substitution of His-385, Cys-62, and Cys-126 in rat Mate1 led to a significant loss of tetraethylammonium transport activity without affecting protein expression at plasma membranes. Similarly, mutation of the corresponding residues in human MATE1 (His-386, Cys-63, and Cys-127) and human MATE2-K (His-382, Cys-59, and Cys-123) also diminished the transport activity. Substitution of the glutamate residues Glu273, Glu278, Glu300, and Glu389 with alanine or aspartate might alter MATE1 function. Glu300Ala, Glu389Ala, and all the aspartate mutants significantly decreased transport of tetraethylammonium. Glu278Asp showed reduced affinity for cimetidine. Both Glu300Asp and Glu389Asp lowered affinity for tetraethylammonium, whereas the affinity of Glu389Asp for cimetidine was fourfold higher than that of the wild-type transporter with about a fourfold decrease in the maximum transport velocity (Motohashi and Inui 2013).

MATEs are electroneutral antiporters of H^+/organic cations and pre-treatment with NH_4Cl stimulated its transport activity. Driving force of MATE is H^+ gradient ($[H^+]_{in}>[H^+]_{out}$). The uptake of tetraethylammonium stimulated by an H^+ gradient was significantly reduced in the presence of a protonophore but was not altered in the presence of valinomycin-induced membrane potential, suggesting that an oppositely directed H^+ gradient drives the transport of tetraethylammonium via MATEs (Tsuda et al. 2007). Usually, the luminal pH is more acidic than the intracellular pH in the cells; the H^+ gradient is maintained via the Na^+/H^+ exchanger and/or ATP-driven H^+-pump (Terada and Inui 2008).

3.5.2 Substrates and Inhibitors of MATEs

Substrates of MATE1 and MATE2-K are typical organic cations including model substrates (such as tetraethylammonium, 4',6-diamidino-2-phenylindole, and 1-methyl-4-phenylpyridinium), clinically used drugs (such as amiodarone, atenolol, cimetidine, cisplatin, diltiazem, metformin, oxaliplatin, procainamide, tetraethylammonium, and topotecan), and toxins (such as paraquat and cadmium) (Motohashi and Inui 2013; Staud et al. 2013; Tanihara et al. 2007; Yang et al. 2017; Yin et al. 2015; Yonezawa and Inui 2011). These compounds are often OCT substrates. Importantly, OCTs are often expressed at the basolateral membrane of corresponding cells, indicating that MATEs work in concert with OCTs to structure an eliminatory pathway of organic cations (Staud et al. 2013). For example, OCT1-MATE1-MDCK cells and OCT2-MATE1-MDCK cells demonstrated higher ratio of basal-to-apical and apical-to-basal metformin transcellular transport than MATE1-MDCK cells, OCT2-MDCK cells, or OCT1-MDCK cells (König et al. 2011). Lamivudine transport is also mediated by both OCT2 and MATE2-K. Trimethoprim inhibited OCT2- and MATE2-K-mediated lamivudine uptake with IC_{50} values of 13.2 and 0.66 μM, respectively, but its IC_{50} on lamivudine transport across OCT2–

MATE1-HEK 293 cell monolayer was 6.9 μM (Müller et al. 2013). These results indicate that the double-transfected cells could be useful for examining the routes by which compounds are eliminated and/or for predicting transporter-mediated drug interaction.

MATEs also transport anionic compounds such as acyclovir, captopril, ganciclovir, and mesna (Staud et al. 2013; Tanihara et al. 2007). Some endogenous compounds such as creatinine, dopamine, E3S, guanidine, N-methylnicotinamide, and thiamine are physiological substrates (Ito et al. 2012a; Kajiwara et al. 2016; Kato et al. 2014; Staud et al. 2013; Tanihara et al. 2007). MATE1 and MATE2-K are very similar, but not completely the same in their substrate specificity. The zwitterionic drugs cephalexin and cephradine are mainly transported by MATE1. Similarly, fexofenadine is substrate of MATE1 but not MATE2-K. Fluoroquinolones such as ciprofloxacin, enoxacin, gatifloxacin, levofloxacin, norfloxacin, pazufloxacin, and tosufloxacin are also transported by rat Mate1 (Ohta et al. 2009). However, ciprofloxacin, levofloxacin, and moxifloxacin are not transported by human MATEs, although they are potent inhibitors of human MATEs (Tanihara et al. 2007; Te Brake et al. 2016). Oxaliplatin is a specific substrate for MATE2-K, and cisplatin is a specific substrate for MATE1, but carboplatin and nedaplatin are not transported by MATE2-K nor MATE1 (Yonezawa et al. 2006).

A series of MATE inhibitors have been identified, most of which are also inhibitors of OCTs. Some compounds such as cimetidine, famotidine, cetirizine, cephalexin, cephradine, imatinib, indinavir, ritonavir, ondansetron, pyrimethamine, mitoxantrone, and topotecan show preferential inhibition on MATEs compared with OCTs (Grottker et al. 2011; Ito et al. 2010; Staud et al. 2013). For instance, cimetidine is inhibitor of OCTs and MATEs, but data from uptakes of five substrates tetraethylammonium, metformin, MPP^+, 4-(4-(dimethylamino)styryl)-N-methylpyridinium, and m-iodobenzylguanidine in OCT1-HEK293 cells and OCT2-HEK293 cells demonstrated that cimetidine is a poor inhibitor with its with K_i values (μM) 101 to 223 and 95 to 146, respectively. These concentrations are markedly higher its clinically reported plasma unbound concentrations (3.6–7.8 μM). On the contrast, data based on MATE1-HEK293 and MATE2-K-HEK293 cells demonstrated that cimetidine is a potent inhibitor of MATE1 and MATE2-K with K_i values (μM) of 1.1 to 3.8 and 2.1 to 6.9, respectively (Ito et al. 2012b), indicating function of MATE1 is strongly inhibited by cimetidine at clinical doses. Data from OCT2-MATE1-MDCK showed that transport of [^{14}C] metformin across cell monolayer was moderately inhibited by the presence of 1 μM cimetidine and almost completely inhibited by the presence of 1 mM cimetidine. Interestingly, the cellular accumulation of [^{14}C] metformin was inhibited by 1 mM cimetidine but increased by 1 μM cimetidine, suggesting that cimetidine at a low concentration inhibits apical MATE1, rather than basolateral OCT2, leading to a marked increase in its cellular accumulation (Tsuda et al. 2009a). However, some compounds such as tetraethylammonium, MPP^+, metformin, disopyramide, procainamide, quinidine, amantadine, and pramipexole have higher affinity for OCT2 than for MATEs (Tsuda et al. 2009a). Moreover, inhibitory effect of MATE inhibitors on human MATE and OCT2 is dependent on substrates. Cimetidine is a much more potent

inhibitor for human MATE1/2-K when metformin is the substrate but acts as an equally potent inhibitor of human OCT2 and human MATE1/2-K when atenolol is the substrate. In OCT2-MATE1-MDCK cells, at clinically relevant concentrations, cimetidine dose-dependently inhibited basal-to-apical flux of atenolol and metformin but impacted their intracellular accumulation differently. Cimetidine is effective only when applied to the basal compartment (Yin et al. 2016). Most of the tyrosine kinase inhibitors, including imatinib, dasatinib, nilotinib, gefitinib, and sunitinib, are potent inhibitors of the MATEs, with IC_{50} values generally in the low micromolar range. Comparison of the unbound maximum clinical concentrations of unbound inhibitors in plasma [I] and IC_{50} values of inhibitors against human MATE1 and MATE2-K showed that imatinib (MATE1 and MATE2-K), gefitinib (MATE2-K), and erlotinib (MATE2-K) exert potent inhibitory effects at clinically relevant concentrations, with $[I]/IC_{50} \geq 0.1$ (Minematsu and Giacomini 2011).

MATE inhibitors often show different inhibitory effect on MATE1 and MATE2-K. In MATE1-HEK293 and MATE2-K-HEK293 cells, when 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide was used for substrate, indinavir, famotidine, ritonavir, and imatinib showed preferential inhibition on MATE1 whose IC_{50} values were lower 10 times compared with MATE2-K. But irinotecan, mitoxantrone, and ondansetron showed similar inhibition on MATE1 and MATE2-K (Wittwer et al. 2013). A report also showed 4 cationic drugs (quinidine, verapamil, procainamide, and ranitidine) had similar affinity for MATE1 and for MATE2-K; 12 cationic drugs (amantadine, cetirizine, chlorpheniramine, cimetidine, desipramine, diltiazem, diphenhydramine, disopyramide, famotidine, imipramine, metformin, and talipexole) had higher affinity for human MATE1 than for human MATE2-K; but affinity of pramipexole to MATE1 was less than that of MATE2-K (Tsuda et al. 2009a).

3.5.3 Pharmacological and Physiological Function of MATEs

MATEs are mainly expressed in luminal membranes of the renal tubules and the bile canaliculi, where they mediate the efflux of substrates taken up by basolateral OCT1 and OCT2, showing important roles in drug disposition.

3.5.3.1 Pharmacokinetic Contributions

In the kidney, MATEs mediate the efflux of drugs from epithelial cells into urine. *Slc47a1*$^{-/-}$ mice have been demonstrated the roles of MATE1 in the pharmacokinetics of several drugs. In *Slc47a1*$^{-/-}$ mice, plasma concentrations of metformin in the plasma, liver, and renal were dramatically increased (Toyama et al. 2011; Tsuda et al. 2009b). The urinary excretion of metformin in *Slc47a1*$^{-/-}$ mice was also

significantly decreased, whose renal secretory clearance was only 14% of wild-type mice (Tsuda et al. 2009b). However, *Slc47a1* heterozygous (*Slc47a1*$^{+/-}$) did not alter pharmacokinetics parameters of metformin in mice, although the level of Mate1 protein was decreased (Toyama et al. 2010). In consistence with this, *Slc47a1*$^{-/-}$ mice showed significantly higher blood lactate and lower pH and HCO_3^- levels following 7-day metformin administration compared with wild-type mice (Toyama et al. 2011). Clinical trial also demonstrated that the blood lactate levels were not affected in patients with the heterozygous MATE variant (Toyama et al. 2011). Pharmacokinetic analysis also demonstrated that diabetic patients carrying heterozygous MATE variant showed similar oral clearance of metformin to patients without heterozygous MATE variant (Toyama et al. 2010). These results indicate that heterozygous MATE variants could little influence the disposition of metformin.

Renal excretion of zwitterionic drug cephalexin is mediated by MATEs. *Slc47a1*$^{-/-}$ mice had higher renal and plasma concentrations of cephalexin than wild-type mice. Total clearance and renal clearance of cephalexin in *Slc47a1*$^{-/-}$ mice were significantly decreased to 68% and 59% of wild-type mice, respectively (Watanabe et al. 2010b).

Apical efflux by the MATE family is considered one of the sites of drug-drug interaction in addition to OCTs at basolateral membrane. Some compounds such as pyrimethamine and cimetidine are specific inhibitors of MATE, although OCTs and MATEs are common in substrate specificity. The affinity of cimetidine for MATE1 and MATE2-K is much stronger than that for OCT2 (Tsuda et al. 2009b) whose IC_{50} values for MATE1 and MATE2-K are 1~10 μM, comparable to plasma concentrations (Tsuda et al. 2009b). However, the K_i value of cimetidine for OCT2 as a competitive inhibitor is 147 μM which is markedly higher than plasma concentrations (Tsuda et al. 2009b), indicating that clinical drug-drug interactions between cimetidine and cationic drugs (such as metformin, procainamide, triamterene, pilsicainide, and varenicline) may be mainly attributed to inhibition of MATEs. In mice, cimetidine significantly increased renal concentration of three substrates (cephalexin, metformin, and tetraethylammonium) and decreased their renal clearance (Ito et al. 2012a). These results confirm that the inhibition of MATEs, but not OCT2, is a likely mechanism underlying the drug-drug interactions with cimetidine in renal elimination.

The antimalaria agent pyrimethamine is a potent inhibitor of MATE1 and MATE2-K, whose IC_{50} values are lower 50 times compared with OCT1 and OCT2 (Ito et al. 2010). In mice, the coadministration of pyrimethamine significantly increased the concentrations of metformin in the kidney and liver, but decreased renal clearance of metformin without affecting plasma concentration of metformin (Ito et al. 2010). In human, oral administration of pyrimethamine significantly reduced the renal clearance of metformin by 23% and 35% at microdose (100 μg) and therapeutic dose (250 mg), respectively (Kusuhara et al. 2011). Pyrimethamine also significantly increased C_{max} and AUC of metformin following therapeutic dose of metformin by 42% and 39% of control values, respectively.

Trimethoprim is a MATE and OCT inhibitor. Clinical trials demonstrated that compared with metformin alone, coadministration of trimethoprim increased C_{max}

and *AUC* of metformin by 22–38% and 30–37%, respectively, and reduced the renal clearance of metformin by 27–33% (Müller et al. 2015; Grün et al. 2013). In healthy volunteers, the concomitant administration of trimethoprim also decreased renal lamivudine clearance by 35%, leading to increases in its plasma *AUC* by 43%, compared with lamivudine alone (Moore et al. 1996). An in vitro study (Müller et al. 2015) showed that inhibition of trimethoprim on MATE1-mediated metformin (*Ki*: 6.3 μM) is fourfold higher than those of MATE2-K (28.9 μM), OCT2 (27.2 μM), and OCT1 (36.7 μM). The inhibitory potency of trimethoprim MATE2-K-mediated lamivudine uptake (IC_{50} = 0.66 μM) was 20-fold higher as compared to OCT2-mediated uptake (IC_{50} = 13.2 μM) (Müller et al. 2013). Assuming a trimethoprim systemic clearance of 8.8 L/h, an unbound fraction of 0.5 (Obach et al. 2008), and near-complete absorption, an average unbound trimethoprim concentration was predicted to 3.3 μM following administration of trimethoprim (200 mg twice daily). Taking these trimethoprim plasma concentrations into account, it is likely that renal clearances of both metformin and lamivudine would be remarkably decreased due to preferential inhibition of MATEs by trimethoprim. Furthermore, coadministration of cephalexin increased C_{max} and *AUC* of metformin by an average of 34% and 24%, respectively, and reduced renal clearance to 14% (Jayasagar et al. 2002) which may be due to inhibition of MATEs by cephalexin.

5-HT_3 antagonist ondansetron is an inhibitor of OCT2 and MATEs, but it shows a much more potent inhibition on MATE1 and MATE2-K than OCT2. The inhibitory potency was 109-fold and 257-fold higher on MATE1 (K_i: 0.035 μM) and MATE2-K (K_i: 0.015 μM) than for OCT2 (*Ki*: 3.85 μM), respectively. A report showed that a clinical relevant dose of ondansetron significantly increased concentration of metformin in the plasma, liver, and kidney wild-type mice, not *Slc47a1*$^{-/-}$ mice, inferring pharmacokinetic interaction of ondansetron with metformin in vivo may be mainly mediated by MATE function (Li et al. 2013b).

MATEs also transport some endogenous compounds including creatinine, dopamine, N-methylnicotinamide, and thiamine (Ito et al. 2012b; Kajiwara et al. 2016; Kato et al. 2014; Staud et al. 2013; Tanihara et al. 2007), indicating the administration of MATE inhibitors may reduce renal excretion of these endogenous substrates. Clinic reports (Ito et al. 2012b; Kato et al. 2014) showed that pyrimethamine significantly reduced renal clearance of thiamine, carnitine, acetylcarnitine, and N-methylnicotinamide by 70~84%, 90~94%, 87~91%, and 70%, respectively. Trimethoprim, another MATE inhibitor, also reduced excreted amount in 24 h urine and renal clearance of N-methylnicotinamide by 19.4% and 19.9%, respectively. Furthermore, decreases of N-methylnicotinamide and metformin renal clearances by trimethoprim correlated significantly (Müller et al. 2015). These results indicate that N-methylnicotinamide and thiamine may be used as endogenous probes for renal drug-drug interactions with involvement of renal cation transporters (Ito et al. 2012b; Müller et al. 2015; Kato et al. 2014).

Renal excretion of creatinine is commonly mediated by renal MATEs, OCTs, and OATs. Pyrimethamine, cimetidine, pyrimethamine, trimethoprim, INCB039110, some kinase inhibitors (such as imatinib, gefitinib, and erlotinib), and fluoroquinolones (such as DX-619 and moxifloxacin) are potential inhibitors of

MATEs, which may at least partly explain these clinical findings that cimetidine, INCB039110, pyrimethamine, and trimethoprim may increase plasma creatinine concentration and reduce renal clearance of creatinine without decreasing glomerular filtration rate (Andreev et al. 1999; Imamura et al. 2011; Zhang et al. 2015).

3.5.3.2 Nephrotoxicity of Cisplatin

In the kidney, cisplatin transport is mediated by OCT2, MATE1, and MATE2-K, indicating that these renal transporters are involved in renal excretion of cisplatin and cisplatin-induced nephrotoxicity. Interestingly, among all platinum agents clinically used, only cisplatin produces severe nephrotoxicity. Kidney-specific OCT2 and MATE1 mediate the transport of cisplatin and are considered to be determinants for cisplatin-induced nephrotoxicity (Yonezawa et al. 2006). Cisplatin and oxaliplatin are both substrates of OCT2 and, therefore, transported into the renal tubular cells. Oxaliplatin is also a good substrate of renal MATEs and is effectively eliminated across the apical membrane into urine. However, cisplatin is a poor substrate of MATEs and accumulates in the kidney cells, resulting in pronounced nephrotoxicity. Transports of carboplatin and nedaplatin are not mediated by OCT2 nor MATEs. The substrate specificity for these transporters may explain differences in the nephrotoxic features of platinum agents (Fig. 3.9).

Roles of MATE1 in the nephrotoxicity of cisplatin have been widely investigated in vivo and in vitro (Li et al. 2013b; Nakamura et al. 2010). Three days after the administration of cisplatin, plasma creatinine and blood urea nitrogen levels were increased, and creatinine clearance was decreased in both wild-type mice and *Slc47a1*$^{-/-}$ mice, compared with vehicle-treated controls. But a significant rise in creatinine and urea nitrogen levels was observed in cisplatin-treated *Slc47a1*$^{-/-}$ mice, compared with wild-type mice. Pharmacokinetic analysis revealed the plasma concentration and renal accumulation of cisplatin to be higher in *Slc47a1*$^{-/-}$ mice than wild-type mice. Furthermore, the combination of a selective MATE inhibitor pyrimethamine or ondansetron enhanced the elevated creatinine and urea nitrogen levels by cisplatin. In vitro study also demonstrated that cisplatin transport is mediated by mouse Oct1, Oct2 and to a less extent, Mate1 (Li et al. 2013b; Nakamura et al. 2010). Several antineoplastic agents such as mitoxantrone, irinotecan, imatinib, dasatinib, nilotinib, gefitinib, and sunitinib were potent inhibitors of the MATEs (Minematsu and Giacomini 2011; Grottker et al. 2011), indicating possibility to potentiate nephrotoxicity of cisplatin when they are coadministrated with cisplatin. It should be noted that most of MATE inhibitors are also inhibitors of OCT2 and renal accumulation of cisplatin is determined by integrated effects of MATE1, MATE2-K, and OCT2. Consequently, OCT2 inhibition using OCT2 inhibitor or OCT2 deficiency may lead to an increase of systemic adverse effects of cisplatin such as hematotoxicity but could be nephroprotective (Tanihara et al. 2009, Ciarimboli et al. 2010). On the contrary, MATE1 and MATE2-K inhibition may increase the nephrotoxicity of cisplatin by increasing its tubular intracellular concentrations (Li et al. 2013b; Nakamura et al. 2010). Therefore, to

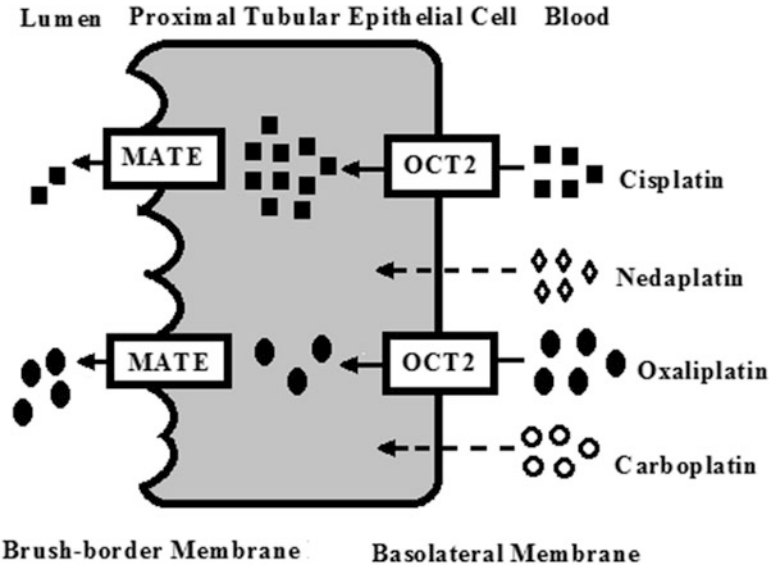

Fig. 3.9 Renal handling of cisplatin, carboplatin, oxaliplatin, and nedaplatin by MATEs and OCT2. Cisplatin is transporter by OCT2 and, to a much lesser extent, by MATE, leading to high accumulation of cisplatin and nephrotoxicity. Carboplatin and nedaplatin are not transported by OCT2 or MATE. Oxaliplatin is a substrate of OCT2 and MATE, but does not induce nephrotoxicity

predict the integrated effect of the triple transporter inhibition on nephrotoxicity is difficult. Clinical report (Sprowl et al. 2013) showed that use of cimetidine did not influence unbound cisplatin plasma clearance. Indeed, as an OCT2 inhibitor, cimetidine showed a nephroprotective effect on cisplatin-induced renal toxicity in mice (Ciarimboli et al. 2010) without efficacy of cisplatin (Sprowl et al. 2013).

3.5.3.3 MATE Polymorphisms and Drug Effects/Toxicity

Although 207 and 206 missense variants for MATE1 and MATE2K have been identified, respectively, most of them with minor allele frequencies. In general, the allele frequencies of missense variants are low and usually do not exceed 2% in different ethnic populations (Nies et al. 2016). In vitro data showed that several missense variants completely lost their function (such as MATE1-Gly64Asp, MATE1-Val480Met, and MATE2K-Gly211Val), which was attributed to an abolished plasma membrane expression of the respective transporter (Kajiwara et al. 2009; Chen et al. 2009b). A series of studies have addressed the association of MATE1 and MATE2 genotypes with pharmacokinetic/pharmacodynamics parameters and treatment outcome of metformin. Among the variants investigated, the MATE1 regulatory region variant *rs2252281* and the intronic variant *rs2289669* were associated with increased metformin response (Becker et al. 2009; Stocker et al. 2013; Tkáč et al. 2013; He et al. 2015). A link between MATE1 *rs2289669*

variant with HbA1c reduction or plasma glucose by metformin has been demonstrated. For example, Tkáč et al. (2013) reported that the patients carrying AA genotype of *rs2289669* have twofold reduction in HbA1c during the first 6 months of metformin treatment in comparison with the patients carrying G allele (GG + GA). A preliminary study (Becker et al. 2009) on 116 diabetic patients with metformin treatment showed that an increase of metformin effect (reduction in HbA1c) of 0.3% per one A-allele of this variant. In another study of Chinese patients following 1-year treatment with metformin, it was found that decreases in HbA1c in patients with AA genotype were significantly stronger than GG and GA genotypes. The patients with AA genotype showed significantly lower renal clearance and renal secretion clearance and higher plasma exposure of metformin than GG and GA genotypes (He et al. 2015). However, several studies did not demonstrate the effect of the *SLC47A1* variant *rs2289669* (Becker et al. 2010; Klen et al. 2014), indicating that other metformin transporters (Becker et al. 2010; Stocker et al. 2013; Klen et al. 2014) and/or nongenetic factors need to be considered as well.

SLC47A1 promoter variant *rs2252281* (g.−66T>C) was also associated with enhanced metformin response in healthy subjects and diabetic patients without affecting pharmacokinetics of metformin (Stocker et al. 2013). However, the *SLC47A2* promoter variant *rs12943590* (g.−130G>A) was reported to be associated with poorer metformin response (Xiao et al. 2016; Stocker et al. 2013) and higher renal clearance and secreted clearance of metformin in the kidney (Stocker et al. 2013; Chung et al. 2013). Moreover, *SLC47A1 rs2289669* was reported to be associated with platinum-induced hematological toxicity (Qian et al. 2016), whereas the variant had no effect on platinum-induced nephrotoxicity (Iwata et al. 2012).

3.6 Peptide Transporters (PEPTs)

3.6.1 General Properties and Tissue Distribution of PEPTs

Mammalian members of the proton-coupled oligopeptide transporter (POT) coded by SLC15 family are integral membrane proteins that mediate the cellular uptake of di-/tripeptides and peptide-like drugs. Main POTs include peptide transporter 1 (PEPT1), peptide transporter 2 (PEPT2), peptide histidine transporter 1 (PHT1), and peptide histidine transporter 2 (PHT2). PEPT1, "professed" intestinal peptide transporter mainly, possess the high capacity and low affinity, while PEPT2, "professed" renal peptide transporter, shows the low capacity and high affinity, indicating that both PEPT1 and PEPT2 may be implicated in disposition of peptide-like drugs and disease process. In addition to di-/tripeptides, both PHT1 and PHT2 mediate the transport of histidine, but their functions are not fully identified (Smith et al. 2013).

PEPTs have 12 TMDs with TMD9 and TMD10 being connected by a long extracellular loop, whose amino- and carboxy-termini are both facing the cytosol. Substrate affinity for PEPT1 and PEPT2 and pH-dependent transport are linked to

the N-terminus of the protein and more specifically to the conserved histidine residues H57, H121, and H260 (Smith et al. 2013). Mutation of His57 and F28Y of PEPT1 alters substrate affinity and pH-dependent transport. R57H of human PEPT2 leads to a complete loss of function. The transporters have several putative glycosylation sites. Murine Pept1 in colon reveals a consistently higher apparent molecular mass of the protein accounting to around 105 kDa, whereas the protein in the small intestine possesses an apparent mass of around 95 kDa. Enzymatic deglycosylation in protein extracts of jejunum and colon yielded an identical protein mass, suggesting that observed Pept1 mass shift was caused by N-glycans (Wuensch et al. 2013). Asparagine residues play pivotal roles in glycosylation. Replacement of six asparagine residues (N) at positions N50, N406, N439, N510, N515, and N532 by glutamine resulted in a decrease of the mouse Pept1 mass by around 35 kDa without affecting its expression (Stelzl et al. 2016). The glycosylation confers resistance against proteolytic cleavage by proteinase K (Stelzl et al. 2017). Functional analysis showed that only substituting N50 with glutamine exhibited a twofold decreased affinity for model dipeptide glycylsarcosine (Gly-Sar), but a 2.5-fold rise in the maximal inward currents compared with the wild-type protein (Stelzl et al. 2016). Importantly, removal of the N50 glycans increased the maximal peptide transport rate in the inward and outward directions (Stelzl et al. 2017).

PEPTs are proton-driven symporters, using the inwardly direct proton electrochemical gradient to drive the uptake of peptides across cell membranes, so they belong to secondary active transporters. The maintenance of the inward H^+ gradient is to require an apical Na^+/H^+ exchanger, in conjunction with basolateral Na^+/K^+-ATPase (Thwaites and Anderson 2007).

3.6.1.1 PEPT1

Human PEPT1 and animal Pept1 are coded by *SLC15A1* and *Slc15a1*, respectively. Human PEPT1 consists of 708-amino acid residues with a 50% overall sequence identity and 70% similarity to human PEPT2. PEPT1 protein is abundantly expressed at the apical membrane of enterocytes from duodenum to ileum of mouse, rat, and human (Jappar et al. 2010; Wuensch et al. 2013; Ziegler et al. 2002). In mouse, Pept1 protein is most abundantly expressed in the small intestine with the highest expression in the duodenum and lower levels toward the colon. In colon, Pept1 protein shows a distinct pattern of distribution with marked differences between proximal and distal segments. Pept1 is essentially absent in proximal colon but becomes detectable again in distal colon (Wuensch et al. 2013). PEPT1 protein is also expressed in brush-border membranes of proximal tubule S1 segments, with progressively weaker expression in deeper cortical regions, inferring that PEPT1 and PEPT2 work in concert to efficiently reabsorb peptides and peptide-like drugs from the tubular fluid (Smith et al. 2013). In addition, PEPT1 is expressed in astrocytes, bile duct epithelial and human nasal epithelium cells, monocytes, macrophages, pancreas, and placenta (Agu et al. 2011; Ayyadurai et al. 2013; Charrier et al. 2006; Smith et al. 2013).

3.6.1.2 PEPT2

Human PEPT2 and animal Pept2 are coded by *SLC15A2* and *Slc15a2*, respectively. PEPT2 protein is primarily localized to brush-border membranes of proximal tubule S3 segments with strong immunostaining of the outer, but not inner, stripe of outer medulla. In the brain, Pept2 mRNA is expressed in rat astrocytes, subependymal cells, ependymal cells, and epithelial cells of choroid plexus. PEPT2 protein has its strongest expression in cerebral cortex, with strong expression also being observed in the olfactory bulb, basal ganglia, cerebellum, hindbrain, epithelial cells of the choroid plexus, and ependymal cells. In addition, Pept2 is exclusively expressed on apical membranes of choroid plexus epithelia in animals. But no evidences have demonstrated expression and function of PEPT2 in endothelial cells of the blood-brain barrier, nor PEPT1 in the brain (Smith et al. 2013). PEPT2 is also expressed in the lung, mammary gland, spleen, and nasal epithelium (Agu et al. 2011; Hu et al. 2014b; Kamal et al. 2008; Smith et al. 2013; Sun et al. 2013). Immune cells such as macrophages and lymphocytes show high expression of PEPT2 (Sun et al. 2013). PEPT2 is also detected in keratinocytes (normal keratinocytes, immortalized keratinocytes, and malignant keratinocytes). In skin tissues, PEPT2 is clearly expressed in epidermal keratinocytes (Kudo et al. 2016).

3.6.2 Substrates and Inhibitors of PEPTs

PEPTs show broad substrate specificity. They can transport almost all dipeptides, tripeptides, and peptide-like drugs which differ in physicochemical characteristics, molecular mass, charge, and polarity. Examples of PEPT substrates include di-/tripeptides and peptide-like drugs such as β-lactam antibiotics (such as cefadroxil), antiviral agents (valacyclovir, alacyclovir, and ganciclovir), L-dopa prodrugs (L-α-methyl-dopa-L-Phe, L-dopa-L-Phe, and D-phenylglycine-L-dopa), polymyxins (colistin and polymyxin B), and 5-aminolevulinic acid (Lu et al. 2016; Thwaites and Anderson 2007). PEPTs also mediate the transport of bacterially produced peptidomimetics including muramyl dipeptide (MDP), N-formyl-methionyl-leucyl-phenylalanine (fMLP), L-alanyl-γ-D-glutamyl-meso-diaminopimelic acid (Tri-DAP), and γ-D-glutamyl-meso-diaminopimelic acid (γ-iE-DAP) (Brandsch 2013; Dalmasso et al. 2011; Dalmasso et al. 2010; Lu et al. 2016; Sun et al. 2013). Gly-Sar, relatively stable against intra- and extracellular enzymatic hydrolysis, is best known reference substrate. It has to be kept in mind that PEPT affinities reported in the literature often show great differences due to tissues, cell types, the experimental protocols (in particular the outside pH and the reference compound), and data processing. For instance, affinities (K_m) of PEPT1 to valacyclovir in Caco-2, *Xenopus laevis* oocytes-PEPT1, MDCK-PEPT1, and CHO-PEPT1 cells show 24-fold varieties, ranging from 0.3 mM to 7.4 mM (Yang et al. 2013). The affinity of cefadroxil for PEPT1 is about twofold lower (i.e., twofold higher K_m) in wild-type mice compared to humanized

PEPT1 mice (Hu and Smith 2016). PEPT1-mediated transport is a proton-dependent process; thus pH also affects PEPT1 activity. The transport of [^{14}C]Gly-Sar at pH 6.0 is approximately five times higher than that at pH 7.5 (Pak et al. 2017). These examples illustrate the need for utmost caution in evaluating affinity constants.

Although PEPT1 and PEPT2 share common substrates, PEPT2 often displays higher affinity for its substrates than PEPT1. For example, ratio of K_i values of Gly-Sar, Ala-Pro, and Ala-Ala-Ala on [^{14}C]-Gly-Sar uptake in Caco-2 cells (human PEPT1) to those in SKPT cells (rat Pept2) are 7.2, 10, and 10, respectively (Knütter et al. 2008). Similarly, valganciclovir shows fourfold higher inhibitory effect on Gly-Sar transport by PEPT1 in Caco-2 cells compared with that by Pept2 in SKPT cells (Sugawara et al. 2000). Generally, PEPT substrates are themselves strong PEPT inhibitors. However, some sartans such as valsartan and losartan are also strong PEPT inhibitor, but they are not transported by PEPTs (Knütter et al. 2009).

3.6.3 Pharmacological and Physiological Function of PEPTs

PEPT1 is mainly expressed in intestine, while PEPT2 is mainly located in the kidney and brain, indicating that PEPT1 and PEPT2 may be implicated in disposition of bi-/tripeptides and peptide-like drugs as well as some disease processing.

3.6.3.1 Intestinal PEPT1 and Intestine Absorption of Drugs

The dipeptides and tripeptides may be transported by intestinal PEPT1 into enterocyte, demonstrating roles of intestinal PEPT1 in absorption of dipeptides and tripeptides. Gly-Sar is a typical PEPT probe. It was reported that Gly-Sar permeability in duodenum of mice was similar to that jejunum, but larger than ileum. In colon Gly-Sar permeability was only 6% of that in the jejunum. The Gly-Sar permeability was positively associated with PEPT1 expression. PEPT inhibitors Gly-Sar, Gly-Pro, carnosine, cefadroxil, valacyclovir, and captopril significantly decreased Gly-Sar permeability in the jejunum. *Slc15a1* deficiency substantially decreased Gly-Sar permeability in the duodenum, jejunum, and ileum. The Gly-Sar permeability in duodenum or jejunum of $Slc15a1^{-/-}$ mice was less than 10% of wild-type mice (Jappar et al. 2010). Uptakes of Gly-Sar into jejunum were reported to $Slc15a1^{+/+}$ mice> $Slc15a1^{+/-}$ mice>> $Slc15a1^{-/-}$ mice, which were in line with levels of intestinal Pept1 protein (Nässl et al. 2011). Pharmacokinetic analysis showed that compared with wild-type mice, *Slc15a1* deficiency resulted in a 2.5-fold reduction in C_{max} and 2-fold reduction in AUC of Gly-Sar following oral administration without affecting disposition of Gly-Sar following intravenous dose (Hu et al. 2008).

PEPT1 can also transport a large number of hydrophilic drugs and is, therefore, responsible for the high levels of oral bioavailability of many pharmaceutical compounds. For example, valacyclovir, an L-valyl ester prodrug of the potent antiviral agent acyclovir, is used for the treatment and prophylaxis of herpes.

Apparent effective permeability (P_{eff}) values of valacyclovir in duodenum, jejunum, and ileum of *Slc15a1*$^{-/-}$ mice were only about 10% of that in wild-type animals. In line with this, Gly-Sar and cefadroxil significantly reduced valacyclovir P_{eff}. Pharmacokinetic analysis showed that *Slc15a1* ablation resulted in three- to fivefold reductions in rate and extent of valacyclovir absorption following oral administration to mice (Yang and Smith 2013; Yang et al. 2013).

Cefadroxil is a preferable substrate of PEPT1. *Slc15a1* ablation significantly decreased the permeability of cefadroxil in the duodenum, jejunum, and ileum of mice, inducing a 20- to 25-fold reduction in C_{max}, a 10- to 15-fold reduction in AUC, and a 3- to 4-fold increase in T_{max} of cefadroxil after oral dose (Posada and Smith 2013a, b), without affecting disposition of cefadroxil following intravenous dose (Posada and Smith 2013b). Intestinal Pept1 also mediates absorption of bestatin and cefixime, leading to occurrence of drug-drug interaction when they are coadministrated. In both intestinal sac preparation of rats and PEPT1-HeLa cells, transports of bestatin and cefixime were significantly inhibited when they were coadministrated. Pharmacokinetic analysis also showed that the plasma concentrations and bioavailability of bestatin and cefixime were decreased and T_{max} values were increased when they were orally coadministrated to rats (Wang et al. 2014).

The characteristics that intestinal PEPT1 mediates absorption of like-peptide drugs have captured the greatest attention to prodrug design to improve oral absorption. Several drugs have been chemically modified for targeting delivery via peptide transporters, including modifications to amine, hydroxyl, or carboxylic acid functionality in the parent drug (Cao et al. 2013; Gupta et al. 2013; Yan et al. 2011; Zhang et al. 2013). Typical examples include valacyclovir and pomaglumetad methionil. The two prodrugs are rapidly absorbed in the intestine via PEPT1 and hydrolyzed to their active metabolites acyclovir (Yang et al. 2013) and LY404039 (Pak et al. 2017), respectively.

3.6.3.2 PEPT2 and Renal Drug Excretion

PEPT2 mRNA and protein are highly expressed in the apical membrane of epithelium in kidney proximal tubule, which is responsible for the reabsorption of small peptides and peptide-like drugs. *Slc15a2*$^{-/-}$ mice have demonstrated roles of PEPT2 in renal excretion of small peptides and peptide-like drugs. For example, cefadroxil undergoes three mechanisms of renal excretion, namely, glomerular filtration, renal tubular secretion, and renal tubular reabsorption. The latter two processes can become saturated at higher plasma concentrations of drug. Generally, cefadroxil is secreted by OATs in the basolateral membrane of the proximal tubule and are reabsorbed by PEPT2 in brush-border membrane of the proximal tubule. Pharmacokinetic analysis showed after an intravenous bolus dose of 1 nmol/g cefadroxil, *Slc15a2*$^{-/-}$ mice exhibited a threefold greater total clearance and threefold lower AUC of the drug compared with wild-type mice. Renal clearance studies demonstrated that the renal Pept2 was almost entirely responsible for the reabsorption of cefadroxil in the kidney of wild-type mice and that renal reabsorption of cefadroxil

in *Slc15a2*$^{-/-}$ mice was almost completely abolished (Shen et al. 2007). Similarly, the clearances of Gly-Sar was about twofold greater in *Slc15a2*$^{-/-}$ mice compared with wild-type mice, whose renal reabsorption was almost abolished in *Slc15a2*$^{-/-}$ mice (Ocheltree et al. 2005). Carnosine, an endogenous dipeptide substrate of PEPT2, plays an important role in many physiological processes. It was reported that systemic clearance of carnosine after intravenous dose was about twofold greater in *Slc15a2*$^{-/-}$ mice. The carnosine uptake was substantially reduced in the kidney of *Slc15a2*$^{-/-}$ mice, leading to eightfold increase in renal clearance. The fractional reabsorption of carnosine in *Slc15a2*$^{-/-}$ mice was only one-fifth of wild-type mice (Kamal et al. 2009). PEPT2-mediated transport is dependent on pH. A clinical trial showed that renal clearance of cephalexin was significantly higher treated with ammonium chloride than that with sodium bicarbonate without affecting other pharmacokinetic parameters. Further study showed that this difference was significant for *SLC15A2*∗2/∗2 but not for *SLC15A2∗1/∗1*, inferring that urinary pH changes may alter the pharmacokinetics of PEPT2 substrates and are more obvious for the *SLC15A2∗2/∗2* (Liu et al. 2011). Chronic kidney disease occurs in most patients with acute intermittent porphyria. During acute intermittent porphyria, 5-aminolevulinic acid accumulates and promotes tubular cell death and tubulointerstitial damage. 5-Aminolevulinic acid and polymyxins are PEPT substrates (Brandsch 2013; Lu et al. 2016). A clinical report showed that carriers of the *SLC15A2∗1/∗1* genotype (higher affinity variant) exhibited worse renal function than carriers of the lower affinity variants *SLC15A2∗1/∗2* and *SLC15A2∗2/∗2*, whose excretion glomerular filtration rate (eGFR) was *SLC15A2*∗1/∗1< *∗1/∗2*< *∗2/∗2*. The change in eGFR over the 10-year period was *SLC15A2*∗1/∗1>∗1/∗2> *∗2/∗2* carriers. At the end of follow-up, 68% of *SLC15A2∗1/∗1* carriers had an eGFR<60 ml/min per 1.73 m^2, compared with 37% of *SLC15A2∗1/∗2* carriers and 15% of *SLC15A2∗2/∗2* carriers. Multiple regression analysis showed that the *SLC15A2∗1/∗1* genotype was associated with an eGFR<60 ml/min per 1.73 m^2 and an annual decrease in eGFR of >1 ml/min per 1.73 m^2. The results gave a clue that a gene variant is predictive of the severity of a chronic complication of acute intermittent porphyria and PEPT2 inhibitors may prevent from porphyria-associated kidney disease (Tchernitchko et al. 2017).

3.6.3.3 PEPT2 and Drug Disposition in the Brain

In the brain, PEPT2 is mainly expressed at the apical surface of choroid plexus epithelia, astrocytes, and neuronal cells. At the blood-CSF interface, function of PEPT2 is to limit the exposure of peptides/mimetics and peptide-like drugs in CSF via transporting these substrates into choroid plexus tissue. Several studies demonstrated that levels of Gly-Sar (Ocheltree et al. 2005), cefadroxil (Shen et al. 2007), and carnosine (Kamal et al. 2009) were significantly increased in CSF, but decreased in plexus tissue of *Slc15a2*$^{-/-}$ mice following intravenous dose compared with wild-type mice. It was also found that the clearance of Gly-Sar from CSF was 3.6

times slower in *Slc15a2*$^{-/-}$ mice than wild-type mice following intracerebroventricular injection of Gly-Sar. Similar alteration in cefadroxil was observed (Smith et al. 2011). 5-Aminolevulinic acid is a precursor of porphyrins. Acute porphyrias are associated with neuropsychiatric symptoms including mood disorders, aggressiveness, insomnia, hallucinations, seizures, and convulsions partly due to 5-aminolevulinic acid accumulation in the brain. It was also reported that 5-aminolevulinic acid concentrations were significantly lower in the choroid plexus, cerebral cortex, kidney, eye, and blood of *Slc15a2*$^{-/-}$ mice following intravenous dose. In contrast to tissue and blood, *Slc15a2*$^{-/-}$ mice had a fivefold greater concentration of 5-aminolevulinic acid in CSF and an eightfold greater CSF/blood concentration ratio. This finding is consistent with the action of PEPT2 that effluxes PEPT substrates from the CSF into choroid plexus epithelial cells. With respect to pharmacodynamics, PEPT2 had a major impact on the ability of *Slc15a2* deficiency to survive the toxic insult following a subcutaneous dose of 5-aminolevulinic acid (4000 mg/kg). The time at which 50% of the animals died in wild-type mice was 21 h, which was significantly larger than that (4 h) in *Slc15a2*$^{-/-}$ mice, providing strong evidence that PEPT2 confers a neuroprotective advantage against 5-aminolevulinic acid toxicity. Neuroprotective roles of PEPT2 were further demonstrated following chronic subcutaneous dose of 5-aminolevulinic acid (500 mg/kg/day for 7 days or 100 mg/kg/day for 30 days). Pharmacokinetic analysis demonstrated that plasma concentrations of 5-aminolevulinic acid in two genotypes following a single subcutaneous dose (100 mg/kg) are similar and concentrations of 5-aminolevulinic acid were 8- and 30-fold greater in the CSF of *Slc15a2*$^{-/-}$ mice at 30 min and 240 min following dose, respectively, which may partly explain the observed pharmacodynamic differences between two genotypes (Hu et al. 2007). Clinic report also showed that children homozygous for the *SLC15A2∗2/2* had poorer motor dexterity and poorer working memory than other PEPT2 genotypes (*SLC15A2∗2/1* and *SLC15A2∗1/1*), which may partly be due to accumulations of 5-aminolevulinic acid and perhaps other peptide-bound amino acids with harmful effects in the brain (Sobin et al. 2015)

3.6.3.4 PEPTs and Inflammatory Response

Commensal bacteria that colonize at the human colon produce significant amounts of oligopeptides such as MDP, Tri-DAP, γ-iE-DAP, and fMLP. These oligopeptides play important roles in the development of mucosal inflammation (Charrier and Merlin 2006). Intestinal PEPT1 mediates transport of these chemotactic peptides into intestinal epithelial cells, leading to initiation of intestinal inflammatory responses including stimulating inflammatory cytokines (such as IL-8 secretion and monocyte chemoattractant protein-1) and increasing neutrophil transepithelial migration (Buyse et al. 2002; Charrier and Merlin 2006; Dalmasso et al. 2010; Vavricka et al. 2004; Wu and Smith 2013). Fortunately, under normal condition, PEPT1 expression is normally restricted to the small intestine, a site in which small bacterial peptide concentrations are low. In colon, PEPT1 expression is very low or absent.

Thus, the profile of PEPT1 expression along the normal digestive tract is such that access of small bacterial peptides to PEPT1 is minimized, limiting their uptake. However, some diseases such as chronic ulcerative colitis, Crohn's disease, and short-bowel syndrome alter this normal expression pattern, leading to significant increases in expression of PEPT1 in the colon (Merlin et al. 2001; Ziegler et al. 2002). It was reported that pathogenic bacteria such as *Escherichia coli* enhanced PEPT1 expression and transport activity in the human colonic epithelial cell line HT29-Cl.19A, which does not normally express PEPT1 (Nguyen et al. 2009). Tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) also significantly upregulated activity and expression of PEPT protein in Caco-2/BBE cells. Treatment with either IFN-γ, TNF-α, or the combination of both significantly also increased activity and protein expression of PEPT1 in proximal and distal colon but not jejunum nor ileum of mice (Vavricka et al. 2006).

Roles of PEPT1 in inflammatory response have been demonstrated (Ingersoll et al. 2012). For example, treatment with 2,4,6-trinitrobenzene sulfonic acid or dextran sodium sulfate induced more severe levels of inflammation in human PEPT1 transgenic mice than wild-type mice. Similarly, intestinal epithelial cell-specific PEPT1 overexpression in villin-human PEPT1 transgenic mice also increased the severity of inflammation induced by dextran sodium sulfate. Antibiotic treatment abolished the effect of PEPT1 overexpression on the inflammatory response in colitis induced by dextran sodium sulfate in both human PEPT1 transgenic mice and villin-human PEPT1 transgenic mice (Dalmasso et al. 2011). On the contrast, dextran sodium sulfate induced less severe levels of inflammation in *Slc15a1*$^{-/-}$ mice than wild-type mice. Antibiotic treatment eliminated the differential expression of inflammatory cytokines induced by dextran sodium sulfate between wild-type mice and *Slc15a1*$^{-/-}$ mice (Ayyadurai et al. 2013). These results indicate that PEPT1-mediated intestinal inflammation is dependent on oligopeptides produced by commensal bacteria. *Slc15a1* knockdown substantially reduced permeability of fMLP in the duodenum, jejunum, and ileum not in colon of mice. In accordance with this, myeloperoxidase activity (a measure of neutrophil migration) in the jejunum of wild-type mice was significantly increased by fMLP and was abolished by glycylglycine (Dalmasso et al. 2010). In Caco2-BBE cells, Tri-DAP induced activation of NF-κB and MAP kinases, leading to production of interleukin-8. The Tri-DAP-induced inflammatory response in Caco2-BBE cells was significantly suppressed by PEPT1-shRNAs. HT29-Cl.19A cells, not expressing PEPT1, were mostly insensitive to Tri-DAP-induced inflammation, but HT29-Cl.19A cells encoding PEPT1 exhibited proinflammatory response to Tri-DAP. Furthermore, Tri-DAP significantly increased keratinocyte-derived chemokine production in colonic tissues from transgenic mice expressing PEPT1 in intestinal epithelial cells (Dalmasso et al. 2010). PEPT1 is also expressed in both immature immune cells and differentiated human macrophages where it transport bacterial peptides (such as fMLP) into macrophages and is involved in the intestinal inflammatory immune response (Charrier et al. 2006). However, a report showed that PEPT1 expression

was consistently reduced under condition of acute or chronic experimental inflammation. Wild-type and *Slc15a1*$^{-/-}$ mice revealed comparable susceptibility to dextran sulfate sodium-induced or 2,4,6-trinitrobenzene sulfonic acid-induced colitis. MDP-induced cytokine expression was also PEPT1-independent. PEPT1 expression levels were also decreased in descending colon of patients with inflammatory bowel disease during acute inflammation, but the *SLC15A1* SNP *rs2297322* was not associated with inflammatory bowel disease susceptibility in the German cohort (Wuensch et al. 2014). These results indicate that roles of PEPT1 in intestinal inflammation need further investigation.

mRNA and protein of PEPT2 are detected in the spleen of mice and human. In comparison to lymphocytes of the spleen, macrophages had higher levels of PEPT2 mRNA. Greater expression of PEPT2 protein was observed in mouse macrophages than lymphocytes (Sun et al. 2013). Similarly, THP-1 macrophages also shows expression of PEPT2 mRNA. In macrophages of mouse spleen, both carnosine and Gly-Sar substantially decreased expression of IL-6 and TNFα mRNA induced by γ-iE-DAP or MDP. In a manner similar to that in splenic macrophages, carnosine decreased the induction of IL-6 and TNFα in THP-1 cells by γ-iE-DAP. The high expression of PEPT2 in the spleen (especially macrophages) shows significant roles of PEPT2 in mediating the innate immune response to bacterially produced chemotactic peptidomimetics (Sun et al. 2013).

3.6.3.5 PEPTs and Cancer

PEPT1 protein is also expressed in some tumors (bladder cancer, colorectal cancer, and hepatocellular carcinoma tissue) and cancer cell lines (such as gastric cancer cells, hepatocellular carcinoma cells, and prostate cancer cells) (Gong et al. 2017; Hagiya et al. 2012; Hagiya et al. 2013; Tai et al. 2013; Viennois et al. 2016), indicating that PEPTs may be implicated in cancer development and its chemotherapeutics. 5-Aminolevulinic acid-based photodynamic therapy is being widely used in cancer therapy owing to the tumor-specific accumulation of photosensitizing protoporphyrin IX (PpIX) after the administration of 5-aminolevulinic acid. A report on five human gastric cancer cell lines (MKN45, MKN28, KKLS, NKPS, and TMK-1) showed that the photodynamic effects on MKN45, MKN28, and KKLS cells were more sensitive than were NKPS and TMK-1 cells. Further study showed that the intracellular PpIX accumulation was positively related with expression of PEPT1 protein. Moreover, overexpression of PEPT1 enhanced the 5-aminolevulinic acid-based photocytotoxicity (Hagiya et al. 2012). Data from a patient who had been subjected to 5-aminolevulinicacid-based photodynamic diagnosis showed the strongest response was observed in papillary pedunculated type tumor, followed by flat type tumor. Immunohistochemical staining demonstrated that papillary pedunculated type tumor, a typical bladder cancer, showed the highest level of PpIX and

strong immunostaining of PEPT1, whereas normal mucosa and flat type tumor showed very weak immunostaining with the PEPT1 antibody (Hagiya et al. 2013).

PEPT1 is expressed in the small intestine at low levels in the healthy colon and upregulated during inflammatory bowel disease. Human PEPT1 transgenic mice were reported to have larger tumor sizes, increase tumor burdens, and increase intestinal inflammation induced by azoxymethane/dextran sodium sulfate compared with wild-type mice. Conversely, *Slc15a1*$^{-/-}$ mice showed significant decreases in tumor number, tumor size, and intestinal inflammation. Proliferating crypt cells were increased in human PEPT1 transgenic mice and decreased in *Slc15a1*$^{-/-}$ mice. Analysis of human colonic biopsy specimens showed increased expression of PEPT1 in patients with colorectal cancer, suggesting that intestinal PEPT1 implicates in development of colon cancer and might be targeted for the treatment of colitis-associated cancer (Viennois et al. 2016).

PEPTs are highly expressed in some cancer, which provides a putative uptake pathway to selectively accumulate anticancer prodrugs in the cancer cells. For example, doxorubicin was conjugated with glycylglycylglycine. The doxorubicin-tripeptide conjugates were demonstrated to be the substrate of PEPT1. Both in vivo and in vitro study showed that antitumor effect of the doxorubicin-tripeptide conjugate on human hepatocarcinoma was comparable to doxorubicin. However, the toxic and side effects of doxorubicin after conjugation were significantly reduced (Gong et al. 2017).

Cancer cells show high expression of PEPTs, indicating that PEPTs may be a suitable target for cancer detection using a peptide-based probe. Positron emission tomography analysis demonstrated that three tumor xenografts (human pancreatic AsPC-1, prostate PC-3, and gastric cancer MKN45 cells) were well visualized with positron planar imaging system after injection of [^{11}C]-Gly-Sar. Tumor-to-blood concentration ratios of [^{11}C]-Gly-Sar increased in a time-dependent manner and were much higher than unity, which were in line with levels of PEPT1 or PEPT2 protein in tumor xenografts. [^{11}C]-Gly-Sar was minimally present in inflammatory tissues due to deficient expression of PEPT1 or PEPT2 protein, but [^{18}F]-fluorodeoxyglucose was still highly accumulated, with the values of the selectivity index being 25.1 and 0.72 for [^{11}C]-Gly-Sar and [^{18}F]-fluorodeoxyglucose, respectively. These results indicate that [^{11}C]-Gly-Sar is a promising tumor-imaging agent and is superior to [^{18}F]-fluorodeoxyglucose for distinguishing between tumors and inflammatory tissues (Mitsuoka et al. 2008).

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Chapter 4
Current Research Method in Transporter Study

Dianlei Wang

Abstract Transporters play an important role in the absorption, distribution, metabolism, and excretion (ADME) of drugs. In recent years, various in vitro, in situ/ex vivo, and in vivo methods have been established for studying transporter function and drug-transporter interaction. In this chapter, the major types of in vitro models for drug transport studies comprise membrane-based assays, cell-based assays (such as primary cell cultures, immortalized cell lines), and transporter-transfected cell lines with single transporters or multiple transporters. In situ/ex vivo models comprise isolated and perfused organs or tissues. In vivo models comprise transporter gene knockout models, natural mutant animal models, and humanized animal models. This chapter would be focused on the methods for the study of drug transporters in vitro, in situ/ex vivo, and in vivo. The applications, advantages, or limitations of each model and emerging technologies are also mentioned in this chapter.

Keywords Membrane-based assays · Cell-based assays · Transporter gene knockout models · Natural mutant animal models · Humanized animal models · Imaging of drug transporters

4.1 General Consideration

As functional membrane proteins, drug transporters are expressed all over the human and animal bodies, and it is well known that transporters play an important role in the absorption, distribution, metabolism, and excretion (ADME) of drugs. Understanding the function of transporters is highly significant in terms of pharmacokinetics, pharmacodynamics, and toxicity of drugs as well as drug-drug interactions (DDIs) in drug development such as discovery, optimization, clinical study, and regulation.

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The selective use of suitable methods for studying drug-transporter interaction is most important in drug development. In recent years, various in vitro, in situ/ex vivo, and in vivo methods have been established for studying drug-transporter interaction. The major types of in vitro models for drug transport studies comprise membrane-based assays (e.g., ATPase assays, membrane vesicular transport studies, and photolabeling assay); cell-based assays including primary cell cultures (e.g., hepatocytes, human bronchial epithelial cells, and brain microvessel endothelial cells); sandwich cultures of primary hepatocytes; immortalized cell lines[e.g., Caco-2, Madin-Darby canine kidney (MDCK), Lilly Laboratories Culture-Pig Kidney Type (LLC-PK1)]; and transporter-transfected cell lines with single transporters [e.g., MDCKII-multidrug resistance associated proteins (MRP) 2, LLC-PK1-multiple drug resistance (MDR) 1, HEK293-organic anion-transporting polypeptides (OATP) 1B1)] or multiple transporters (such as MDCKII-MRP2/OATP1B1). In situ/ex vivo models comprise isolated and perfused organs or tissues (e.g., isolated perfused intestine, lung, liver, brain or kidney). In vivo models comprise transporter gene knockout models, natural mutant animal models, and humanized animal models.

This chapter would be focused on the methods for the study of drug transporters in vitro and in vivo. The applications, advantages, or limitations of each model and emerging technologies (e.g., imaging of drug transporters) are also mentioned in this chapter.

4.2 In Vitro Models

4.2.1 Membrane-Based Assays of ABC Transporters

Membrane vesicles prepared from cells expressing transporters are suitable to study the function of the ATP-binding cassette (ABC) as well as the solute carrier (SLC) transporters and to identify their substrates or inhibitors. There are two classical membrane-based assays: the ATPase assay and the membrane vesicular transport (uptake) assay.

4.2.1.1 ATPase Assays

The ABC family transporters use ATP as energy source to transport substrates across the plasma membrane. Since the functions of the transporter proteins are often correlated to ATP hydrolytic activity, the functions may be assessed indirectly by ATP hydrolytic activity (Sarkadi et al. 1992; Ambudkar et al. 1992).

The most common method to measure ATPase activity is the colorimetric detection of liberated inorganic Pi (Williamson et al. 2007). The ATPase assay does not require the use of radioactivity or special instruments to analyze the drug, so it is suitable for high-throughput application. Experimentally, the substrates at a

single concentration or a concentration range are incubated, respectively, with cells or tissues expressing ABC transporters such as P-glycoprotein (P-gp), MRP, and breast cancer resistance protein (BCRP). The colorimetric determination of inorganic phosphate produced by ATP cleavage during transport can reflect the activity of drug transport.

There are some limitations of ATPase assay. Firstly, for its indirect measurement of transport, the assay is unable to distinguish transporter substrates or inhibitor directly. Therefore, the method is not a suitable tool for identifying the transport's functions. For example, some drugs showed different effects on the Pi release at different concentrations (Polli et al. 2001; Markowitz et al. 2002). Secondly, for some substrates and inhibitors, ATPase activity is not consistent with the rate of transport, and ATPase assays may produce false-positive or false-negative results. Substrates transported at a slow rate by ABC transporters may not stimulate ATPase activity to a detectable extent of inorganic phosphate and thus produce false negatives. For example, cyclosporine A (CsA) and paclitaxel, the P-gp substrates, which have been demonstrated with other methods (Goldberg et al. 1988), showed different results in the P-gp ATPase activation assay.

4.2.1.2 Membrane Vesicular Transport Studies

The vesicular transport assay generally uses inside-out oriented membrane vesicles, to characterize the transporter kinetics of a substrate by quantifying its accumulation into the vesicle. The inside-out membrane vesicles can be used for investigation of concentration-transport relationship, estimation of the kinetic parameters (such as K_m, K_i, and V_{max}), and the characteristics of inhibitors. Compared to ATPase assays, the inside-out membrane vesicles assays have the mechanistic advantage of exact transport of drug or substrate across the cell membrane.

Experimentally, the inside-out membrane vesicles could be prepared from various sources through a series of homogenization and centrifugation steps. The inside-out membrane vesicles may come from different sources such as transfected insect cells (e.g., Sf9 or Sf21) and selected and cDNA-transfected mammalian cell lines [e.g., HeLa, V79 hamster, human embryonic kidney (HEK293), and MDCK], and isolated primary cells or tissues (Sharom et al. 1999). The plasma membrane vesicles that overexpress the bile salt export pump (BSEP) or MRP2, MRP3, and MRP4 may be used to investigate whether drug candidates affect transporter function in hepatocytes and cause drug-induced liver injury (DILI) (van Staden et al. 2012). The vesicles prepared from tissues (such as intestine, kidney, and liver) could also be applied to characterize the tissue-specific transporter proteins (Tamai et al. 1988; Yasumiba et al. 2001; Zalups et al. 2004). However, the contamination with the opposite membrane domain should be taken into consideration. Therefore, when uptake of substrate by membrane vesicles in the presence of ATP is documented, incubations without ATP are often served as negative controls (Doige and Sharom 1992).

After a certain time of incubations, a rapid filtration using glass fiber or nitrocellulose membranes and washing at 4 °C was done to remove any remaining external compound (Imaoka et al. 2007; Hu et al. 2010), and then the substrate trapped in the vesicle can be quantified via analysis such as LC-MS/MS, HPLC, fluorescence detection, or scintillation counting, depending on the detection equipment available for analysis. Inhibitors can be identified by co-incubating the compounds with a reporter probe substrate which has always been an efficient method to characterize the inhibitors (Stieger et al. 2000; Zelcer et al. 2003).

There are some advantages of vesicle-based assays. Firstly, the method can be adapted to a multi-well plate format for high throughput. Secondly, the commercial vesicles can eliminate the variability in different lab and preparation as they were generated in large quantities and stored in liquid nitrogen or at −80 °C without loss of activity. However, the major limitation of vesicle assay may lead to false-negative results when it is used for hydrophobic substrates, due to its non-specific binding and leakage from the vesicles. But its limitation could be decreased to minimal effect by rational study design, and more study designs such as consistent procedures, compound solubility, incubation time, etc. should also be taken into consideration (Brouwer et al. 2013).

4.2.2 Cell-Based Assays

The use of immortalized cell lines or primary isolated cells has many benefits including the intact cellular architecture, functional membrane, cytoplasmic elements, and cotransporting ions, which could produce more definitive results in the characterization of transporter functions. However, these immortalized cell lines and primary cells often lack expression of some special transporter protein, and the transport assays are usually conducted in stable cell lines or recombinant cell lines expressing the special transporter(s) (Zhang et al. 2009; Keppler 2005). The cell lines such as MDCKII, LLC-PK1, Chinese hamster ovary (CHO), HEK293, and HeLa cells were commonly taken as transporter-transfected cells.

4.2.2.1 Transport Assays

Transport assays require the immortalized or primary cells or transporter-transfected cell lines that form monolayers with tight junctions and polarized transporter location. The characteristics of drug transport (passive transport or transporter-mediated transport) are documented via analyzing the bidirectional drug transport across the monolayer.

The targeted cells are cultured in a permeable membrane in a cell culture system until they reach confluence and full differentiation, and the polarization of the cells defines the apical and the basolateral liquid compartment, which are directly in

contact with the respective membrane domain of the cells. The drug transfer between the two compartments is a sum of diffusion, passive permeability, and interactions with transporter expressed in the membrane of cells.

The typical bidirectional transport experiments are as follows: firstly, cells are seeded on the permeable membrane to form a monolayer; secondly, tightness of cell monolayer should be validated via measuring the paracellular flux of a low-permeability compound such as fluorescein; thirdly, the rate of transport of the drug in the apical to basolateral ($A-B$) and basolateral to apical ($B-A$) directions was measured. The apparent permeability (P_{app}) may be calculated using the following equation.

$$P_{app} = \frac{\Delta Q}{\Delta t \times A \times C_0} \tag{4.1}$$

where ΔQ, A, and C_0 are cumulative transported amount of drug during changed time (Δt), area of membrane, and initial concentration, respectively. The ratio (ER) of basolateral to apical ($P_{app,\ B-A}$) and apical to basolateral ($P_{app,\ A-B}$) is also estimated.

4.2.2.2 Uptake Assay

Studies using in vitro cell model are important to characterize the drug uptake, tissue distribution mechanisms, and the potential transporter interaction. The uptake kinetic parameters (K_m, V_{max}, and IC_{50}) of transporter substrate or inhibitors for transporter are generally determined. Uptake transporters are main members of SLC superfamily such as *L*-type amino acid transporter (LAT), peptide transporters (PEPTs), sodium-glucose cotransporters (SGLTs), glucose transporters (GLUTs), monocarboxylate transporters (MCTs), organic anion-transporting polypeptides (OATPs), organic anion transporters (OATs), and organic cation transporters (OCTs). These transporters are either passive facilitators or active pumps.

Whether a drug is the substrate or inhibitor of a transporter may be directly identified via measuring drug uptake in the tested cells in the presence of special transporter substrate or inhibitor. The cell lines overexpressing transporter genes or primary isolated cells (such as hepatocytes and sandwich-cultured hepatocytes) or the immortalized cell lines may be selected according to the purpose of the study. For kinetic study, the assay should be done in different drug concentrations.

For substrate characterization, these assays are often performed using cell lines overexpressing transporter genes and wild-type cell lines. Drug affinity to uptake transporter could be assessed by its uptake rate or the amount in the cells. In most cases, special transporter inhibitor is used for identifying transporter substrate (Feng et al. 2008). Using a fluorescent or a radiolabeled compound as a substrate, the uptake assay is a high-throughput assay for transporter inhibitor screening (Gui et al. 2010).

4.2.2.3 Efflux Assays

Efflux assays are also suitable for the screening of compounds that interfere with efflux transporters. These assays can be performed using cell suspensions and cell monolayers. The drug efflux transporters are mainly ABC transporters such as P-gp, MRPs, BCRP, and BSEP.

The cells are pre-loaded with the special substrate or interested compound. Then, the residual amount of substrate in the cells is measured following incubation with the indicated agents. In general, efflux transporter inhibitors decrease efflux of substrate from cell, leading to higher cellular accumulation of substrate compared with control cells. On the contrast, efflux transporter inducers or activators enhance efflux of substrate from cells. Thus the method is often used to characterize inhibitors, inducers, or activators of efflux (Gameiro et al. 2017). Fluorescent compounds are often served as their substrates, because the cellar amount of these fluorescent probes are detected directly using flow cytometry or any fluorescence detectors. Commonly used P-gp substrates include rhodamine 123, doxorubicin, daunorubicin, and calcein-AM (Palmeira et al. 2012).

4.2.2.4 Commonly Used Cell Systems

4.2.2.4.1 Caco-2 Cells

Caco-2, a human colon adenocarcinoma cell line which is similar to human small intestinal enterocytes, was developed by the Sloan Kettering Institute for Cancer Research through research conducted by Dr. Jorgen Fogh (Fogh 1975). The cells cultured under specific conditions become differentiated and polarized so that their phenotype, morphology, and function are similar to the enterocytes lining the small intestine (Pinto et al. 1983; Hidalgo et al. 1989).

Caco-2 cells form tight junctions and microvilli, and express a number of enzymes and transporters, which make it an excellent model for studying transmembrane processes (Hayeshi et al. 2008). This model has been widely used to study intestinal permeability, absorption, and transport involvement (Giacomini et al. 2010) and successfully used in the pharmaceutical industry (Artursson 1990) and fully validated for regulatory submissions in the pharmaceutical industry (Elsby et al. 2008).

Although there is a correlation established using Caco-2 model between a number of drugs and transporter-mediated drug absorption, the limitations of Caco-2 model include long differentiation time (about 3 weeks), the variability in permeability, and transporter expression level in different laboratories. Therefore we need some special marker for permeability as control in the experiment and carefully notice the expression level of transporters in individual laboratory. In addition, the multiple transporters expressed in Caco-2 cells are not suitable for studying the specific transporter function. To overcome the limitation, Caco-2 sublines with single or

double functional transporters have been reported to study the special transporter using the specific gene knockdown technologies such as zinc finger technology (Pratt et al. 2012), small interfering RNA, and single hairpin RNA. Those methods give the specific interpretation of transporter function on membrane transport and permeability (Sampson et al. 2015).

4.2.2.4.2 Hepatocytes

The freshly isolated or cryopreserved hepatocytes have been used to study uptake, metabolism, and hepatic clearance of drugs (Soars et al. 2009). Fresh hepatocytes are isolated by collagenase perfusion of livers from rat or human donors (LeCluyse et al. 1996). The hepatocytes should be dispensed in special medium or buffer. Suspended hepatocytes should be used immediately or short time after preparation for their viability decreasing over time. The primary hepatocytes from rat or human could be used in suspension assays to conduct a time-dependent uptake or inhibition study, invariably using a known probe substrate and with known inhibitors or at low temperature (to measure the uptake kinetics) (Li et al. 2008; Bi et al. 2012). Otherwise, for the lack of specific inhibitors or substrates, it may be difficult to determine which individual isoforms of specific uptake transporters are involved in uptake of compounds. Suspended hepatocytes are often used to determine the role of transporters in hepatic uptake because plated hepatocytes exhibit decreased transporter function in the following culture (Ishigami et al. 1995).

Though suspended primary hepatocytes have the ability to reproduce hepatic uptake function, they have the inability to access the canalicular efflux because canalicular transporter proteins are internalized after isolation (Bow et al. 2008). For this reason, a sandwich-cultured hepatocyte (SCH) technique has been developed. In this system, primary hepatocytes are placed between two layers of a gelled matrix and in a sandwich configuration. When freshly isolated or cryopreserved hepatocytes are cultured in SCH culture system for a period of 4 days (rat SCH) or 6–7 days (human SCH), hepatocyte polarization and canalicular networks are regenerated which are vital for the direction to transport. The hepatocyte architecture, including tight junctions, the formation of intact canalicular networks, and the polarized excretory function are kept close to the physiological status over a longer period of time (Swift et al. 2010). As a result of these features, the SCH not only are a valuable tool to evaluate both uptake and efflux hepatobiliary transporters but also are very useful in drug discovery and development to assess biliary in vitro for its good correlation between in vitro and in vivo (Abe et al. 2009; Yang et al. 2016). In addition to the expression of functional actively basolateral and apical transporter proteins, SCH also express some metabolism enzymes (De Bruyn et al. 2013). Since the SCH model reconstitutes the liver's metabolizing enzymes and transporters, it can be used to evaluate the induction of transporters and enzymes by xenobiotics and to understand interspecies difference in hepatobiliary transporters and enzymes. In addition, due to the well-maintained regulatory machinery for transporters, such as Farnesoid X receptor, SCH has been used to study the induction of transporters at the

expression and function levels (Zhang et al. 2017; Guo et al. 2018). Kinetic parameters such as uptake and efflux clearances in SCH can be estimated through mechanistic pharmacokinetic modeling (Guo et al. 2016). The parameters obtained can be incorporated into a PBPK model for in vivo human pharmacokinetics and DDI prediction (Jones et al. 2012; Varma et al. 2012).

The transporters and enzymes in hepatocytes in suspension or SCH are dependent in culture conditions and vary from batch to batch (Shitara et al. 2003; Swift et al. 2010). Recently, immortalized hepatic cell lines and stem cell-derived lines have been used. For example, HepaRG cell lines have been used to study the nuclear regulation receptors, which can express the liver-specific glycolytic enzymes and induce the major CYP450 enzymes (Guguenguillouzo and Guillouzo 2010). Meanwhile, the modifications in the SCH system become increasingly like in vivo and continue to be explored. A three-dimensional (3D) culture model has been developed in which hepatic phenotypes are maintained for extended periods of time (Lauschke et al. 2016). It was reported that the primary human hepatocyte (PHH) spheroids remained phenotypically stable and retained morphology, viability, and hepatocyte-specific functions for culture periods of at least 5 weeks (Bell et al. 2016). 3D PHH spheroids express levels of phase I drug-metabolizing enzymes (e.g., CYP2C8, CYP2C9, CYP3A4, and CYP2D6), phase II drug-metabolizing enzymes (e.g., GSTT1 and UGT1A1), drug and bile transporters (ABCB11 and ABCC1 and SLCO1B1), and other genes with importance for hepatic functions, whose expressions in the 3D PHH spheroids were close to the levels in the corresponding freshly isolated cells. Meanwhile, PHH spheroids also highly express some physiologically important transporters such as BSEP, Na^+-taurocholate cotransporting polypeptide (NTCP), OATP1B1, OATP1B3, and the phosphatidylcholine transporter (ABCB4). Therefore, the development and characterization of the 3D PHH spheroid model constitute a promising method in vitro for drug-transporter study (Bell et al. 2017).

4.2.2.4.3 MDCKII Cells

The MDCKII cell line is a polarized cell line with tight junctions and separation of apical and basolateral membranes. The MDCKII cells themselves lack expression of some drug transporters, which provides an additional advantage to MDCKII cell lines to transfect multiple transporters. The MDR1-transfected MDCKII cells are often used to characterize the substrates and inhibitors of P-gp and to predict the permeability of P-gp substrates across blood-brain barrier (BBB) (Wang et al. 2005). MDCKII cell lines transfected with MDR1, MRP1, or MRP2 transporters were used to characterize the transport of etoposide, demonstrating that P-gp and MRP2 were involved in the intestinal secretory transport of etoposide (Guo et al. 2002). MDCKII cells expressing multiple transporters have been developed such as MDCKII-OATP1B1/MRP2 MDCKII-OATP2/MRP2, MDCKII-OATP1B3/MRP2, OATP1B3/MDR1, OATP1B3/MRP2, and OATP1B3/BCRP (Cui et al. 2001; Sasaki et al. 2002, 2004; Letschert et al. 2005; Saito et al. 2007). MDCKII cell

lines stably expressing recombinant OATP1B1, OATP1B3, and OATP2B1 in the basolateral membrane and ABCC2 in the apical membrane were also generated, which characterized high rates of vectorial transport of bromosulfophthalein, cholecystokinin peptide, and estrone 3-sulfate. The quadruple-transfected MDCKII cells may be useful for the identification of substrates and inhibitors, including drug candidates, undergoing uptake and secretion by human hepatocytes (Kopplow et al. 2005). The MDCKII-OATP1B1-CYP3A4-UGT1A1-MRP2 cell line was used to investigate interplay of bosentan metabolism and transcellular transport (Fahrmayr et al. 2013).

MDCKII cell lines have the drawback of highly expressing some endogenous transporters. A report showed that Bcrp1 transfection significantly upregulated expression of OCT2 in MDCKII cells, leading to enhancement in the uptake of several organic cations (Pan et al. 2010). Therefore, transport studies involving transfected MDCKII cell lines need an additional study for comparing with wild-type MDCKII cell lines to estimate endogenous transporters on the transport of drugs (Gartzke and Fricker 2014).

4.2.2.4.4 HEK293 Cells

HEK293 cells were generated by transformation of human embryonic kidney cell cultures with sheared fragments of adenovirus type 5' DNA and were first described in 1977 (Graham et al. 1977). The major advantage of the cell lines is the minimal expression of endogenous transporters and metabolic enzymes, which made them being a good host for expressing exogenous transporters (Hilgendorf et al. 2007).

HEK293 cells stably transfected with OAT1, OAT3, and OCT1/2 transporter gene were used to study the effect of 22 antituberculosis drugs on uptake of substrate for OATs and OCTs. The results demonstrated that several antituberculosis drugs showed significant inhibitory effect on hepatic uptake of OAT1- and OAT3-mediated para-aminohippurate and OCT2-mediated N-methyl-4-phenylpyridinium acetate and OAT-mediated zidovudine (Parvez et al. 2017).

HEK293 or CHO cells stably transfected gene of OCT1, OCT2, or their allelic variants were used to investigate the effects of OCT1 and OCT2 genetic polymorphisms on the uptake of ranitidine and on its potency to inhibit uptake of other drugs. The results demonstrated that genetic polymorphisms of OCT1 strongly affected ranitidine uptake and that ranitidine potentially caused DDIs when coadministrated with OCT1 substrates (Meyer et al. 2017).

4.2.2.4.5 HeLa Cells

HeLa cells, an oldest immortalized cell line, were derived from cervical cancer cells taken from a patient named Henrietta Lacks in 1951 (Scherer et al. 1953) and are most frequently used in drug research (Rahbari et al. 2009). Zhang et al. (1998) investigated the interaction of an array of organic cations and other compounds in

transiently transfected hOCT1 HeLa cells via determining K_i values for inhibition of [^{14}C]-tetraethylammonium (TEA) transport. They found that the maximal expression of hOCT1 occurred at approximately 40 h following transfection. Organic cations, including clonidine, quinine, quinidine, and verapamil, significantly inhibited [^{14}C]-TEA uptake. Neutral compounds corticosterone and midazolam also showed potent inhibition on [^{14}C]-TEA uptake (Zhang et al. 1998). In HeLa cells transiently expressing allelic variants of OATP1B3 genes, it was reported (Schwarz et al. 2006) that compared with wild-type OATP1B3, OATP1B3∗4, OATP1B3∗6, and OATP1B3∗7 exhibited significantly lower uptake activity of cholecystokinin 8, accompanied by remarkably lower V_{max} values without affecting affinities to cholecystokinin 8. Cell surface biotinylation suggested that altered plasma membrane expression may contribute to reduced transport activity associated with the OATP1B3∗6 and ∗7 variants (Schwarz et al. 2006).

4.2.2.4.6 CHO Cells

CHO cells, derived from the ovary of the Chinese hamster, have been used for transfection of uptake and efflux transporters. CHO transfected with OATP1B1, OATP1B3, and HEK293 cells transfected with OATP2B1 have been used for gemfibrozil-statin interaction at the level of active hepatic uptake. The results showed that only the high-affinity component was inhibited by gemfibrozil, and recombinant OATP1B1-, OATP2B1-, and OATP1B3-mediated fluvastatin transport was inhibited to 97, 70, and 62% by gemfibrozil, respectively (Noé et al. 2007). The intracellular accumulation of oseltamivir and its carboxylate metabolite oseltamivir carboxylate in CHO-hPEPT1 cells and CHO cells were always similar under a variety of experimental conditions. Furthermore, neither oseltamivir nor oseltamivir carboxylate was capable of inhibiting GlySar uptake in the CHO-hPEPT1 cells (Poirier et al. 2012). These results demonstrated that neither oseltamivir nor oseltamivir carboxylate was substrate of hPEPT1.

4.2.2.4.7 LLC-PK1 Cell

LLC-PK1 cell lines derived from epithelial cells of porcine kidney proximal tubules are also widely used for transfection of uptake and efflux transporters. Yamazaki et al. (2001) investigated the species difference in the substrate specificity to P-gp by measuring bidirectional transcellular transport of compounds using MDR1 and Mdr1a-transfected LLC-PK1 cells (Yamazaki et al. 2001). In LLC-PK1 cells, it was found that acidification of the extracellular pH or alkalization of the intracellular pH significantly decreased uptake of quinidine (Matsui et al. 2018). Further study showed that the cellular uptake of quinidine from the apical side was much greater than from the basolateral side and that the apical efflux of quinidine from LLC-PK1 cells was increased by acidification of the extracellular pH. Lipophilic cationic drugs also significantly reduced uptake of bisoprolol in LLC-PK1 cells. In vivo study

showed that alkalization of the urine pH markedly decreased renal clearance of bisoprolol in rats (Matsui et al. 2018). These results suggested that LLC-$PK_{1\ and}$ renal tubular epithelial cells of rats express a H^+/lipophilic cation antiport system, which may be responsible for renal tubular secretion of some cationic drugs including bisoprolol in rats.

4.2.2.4.8 Oocytes

The oocyte (or egg cell), from the South African clawed frog *Xenopus laevis*, belongs to the largest single cells in the animal kingdom. Six different stages (stages I to VI) of development have been classified by Dumont according to size (Dumont 1972). Therefore, care should be taken to use only oocytes of the same stage in the process of experiment. Oocytes can be obtained from the ovary of female *X. laevis* frogs (Smith et al. 1991).

The oocytes have the ability to translate foreign genetic information into functional proteins efficiently, which are used as a functional expression system for transporter protein. Similarly to the cDNA-transfected mammalian cells, the oocytes expressing transporters have been used to characterize the interactions between transporters and their substrates and inhibitors, to measure the kinetic parameters for transporter substrates or inhibitors, to evaluate transporter polymorphisms, and to identify species differences in transporter function (Cindy et al. 2007). Renal transporters (such as OAT1, OAT3, OCT1, and OCT2), peptide transporters, and ABC transporters (such as BCRP) have been extensively characterized in the *Xenopus* oocyte system (Nakanishi et al. 2003; Uwai et al. 2004).

The drawbacks of oocyte expression system include the following: (1) transient expression of the transporter, which usually lasts no longer than 14 days; (2) seasonal variations in the quality of the oocytes. As the *Xenopus* frog is a poikilothermic animal, its oocytes are kept best at lower temperature; (3) low to medium throughput assays; (4) cDNA that has to be transcribed, capped, and polyadenylated in vitro unless it is directly injected into the nucleus of the oocytes; (5) requirement of microinjection skill for transfecting cDNA or mRNA and for efflux transporter studies; (6) when oocytes are used to investigate the regulation of expressed proteins, they may have different signaling pathways and specificities as genuine tissue or cells (Sigel 1990).

4.2.2.4.9 Brain Microvessel Endothelial Cells (BMECs)

The structure responsible for the formation of the BBB is BMECs that are bound together by tight intercellular junctions and form a barrier from blood to brain. Many methods have been reported to isolation and culture of cerebral microvessel cells to form an in vitro BBB model (Audus and Borchardt 1986; Qian et al. 1999; Sun et al. 2006; Zhang et al. 2003).

The protocols of primary BMEC transport experiments are as follows. Firstly, BMECs are isolated and grown in culture to monolayers. Secondly, these endothelial cell monolayers are characterized morphologically with electron microscopy, histochemically for brain endothelium enzyme markers (such as alkaline phosphatase and γ-glutamyl transpeptidase), and by immunofluorescence to detect factor VIII antigen, an exclusive endothelial antigen. Thirdly, the transendothelial transport function and expression are measured. Uptake of P-gp substrate rhodamine 123 and Western blot assay are often used to determine the P-gp function and expression in the BMECs (Audus and Borchardt 1986; Liu et al. 2009; Zhang et al. 2003).

mRNA expressions of some ABC transporters in the HBECs were detected using quantitative real-time PCR. In the isolated BMECs from human, rat, mouse, pig, and cow, it was found that several ABC transporters, including ABCA2–4, ABCB4, ABCB6–8, ABCB10, ABCC3, ABCC6, ABCC10, and ABCE1, are highly expressed in BMECs in one or more species (Warren et al. 2009).

Confocal microscopy and Western blot analysis demonstrated that OCT1 and OCT2 were localized in cultured adult rat BMECs. Silencing of OCT1, OCT2, or both together significantly decreased cellular uptake of N-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) by about 53%, 60%, or 91%, respectively. Amantadine also competitively inhibited MPTP uptake. The results indicated that OCT1 and OCT2 are important for MPTP transfer across the BBB and amantadine could reduce the BBB transfer of MPTP and MPTP-induced dopaminergic toxicity in rodents (Lin et al. 2010).

The BMECs may be used to investigate effects of some diseases on function and expression of drug transporters. In primarily cultured rat BMECs P-gp, it was found that 72-h incubation with insulin (50 mU/L) significantly increased P-gp function and expression and that the induced effect could be blocked by insulin receptor antibody, insulin receptor tyrosine kinase inhibitor I-OMe-AG 538, PKC inhibitor chelerythrine, and NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) ammonium, but not phosphatidylinositol 3-kinase (PI3K)/Akt inhibitor LY294002, indicating that insulin regulated P-gp function and expression through signal transduction pathways involving activation of PKC/NF-κB but not PI3K/Akt pathway (Liu et al. 2009). Patients with epilepsy often do not respond to clinically established antiepileptic drugs, which is attributed to overexpression of P-gp. A report showed that 60-day exposure of four typical antiepileptic drugs(phenobarbital, phenytoin, carbamazepine and valproic acid) to BMECs in a gradient concentration manner significantly increased expression and function of P-gp. The data supported the possibility of involvement of chronic drug exposure in change of P-gp function and level, which may contribute to antiepileptic drugs refractoriness (Yang et al. 2008).

4.2.2.4.10 Human Bronchial Epithelial Cells (HBECs)

HBECs can be extracted from bronchi procured locally through cooperation of surgeons and pathologists in accordance with relevant institutional, local, and national regulations. HBECs were cultured on culture dishes coated with human airway epithelial cell in bronchial epithelial growth medium (BEGM) to maintain at an air-liquid interface (ALI) represent a leap toward the in vivo biology, and was used to support growth and differentiation on porous supports (Fulcher et al. 2005).

Human epithelial barrier of the lungs in vitro models have been developed recently in response to ethical concerns regarding the use of laboratory animals (Sporty et al. 2008). 16HBE14o- cells are normal human bronchial epithelial (NHBE) cells; when cultured as monolayers on permeable supports at an air-liquid interface, it provides in vitro representations of the absorption barrier of the upper airways morphologically close to the native bronchial epithelium while exhibiting similar permeability properties (Forbes et al. 2003; Lin et al. 2007). In addition, many ABC transport systems such as P-gp, MRP1–9, and BCRP that are found in the human lung have been characterized in 16HBE14o- cells. MRPs are known to regulate the efficacy of detoxification by transporting toxic compounds, including chemotherapeutic drugs, out of cells. Due to their role in efflux of glutathione (GSH)-conjugated drugs, MRPs can also regulate cellular oxidative stress, which may contribute to pulmonary pathogenesis. The high expression of MRP1 was found on the basolateral membrane of ciliated, mucous-producing, and basal cells in the lungs (Scheffer et al. 2002), which suggested the important role of MRP1 in the pathophysiology of chronic obstructive pulmonary disease (COPD) (van der Deen et al. 2006). 16HBE14o- cells also showed a strong immunohistochemical staining for MRP1. The functionality of MRP1 in normal lung cells was demonstrated by the decreased efflux of the MRP substrate carboxydichlorofluorescein (CDF) in the presence of the MRP inhibitor MK-571 (Lehmann et al. 2001). The 16HBE14o- cell lines were used to evaluate the effect of cigarette smoke and drugs commonly used in the treatment of COPD on MRP1 activity in the bronchial epithelium. It was reported that exposure of cigarette smoke extracts diminished the efflux of CDF out of cells (van der Deen et al. 2007), which may be attributed to a direct interaction of cigarette smoke components with the transporter. Budesonide enhanced the intracellular accumulation of CDF in 16HBE14o- cells, which was reversed by formoterol although formoterol on its own had little effect on MRP1 activity (van der Deen et al. 2008). On the contrast, ipratropium and N-acetylcysteine decreased the CDF accumulation, suggesting the two compounds stimulated MRP1-mediated efflux.

Allyl isothiocyanate (AITC), a hydrolysis product of the glucosinolate sinigrin, has significant antimicrobial activity and possesses potential anticancer activity such as colorectal and bladder cancers. A report showed that AITC concentration dependently increased expressions of MRP1 protein and mRNA as well as increased MRP1-dependent efflux of CDF in 16HBE14o- cells (Wang et al. 2014). Further investigation indicated that AITC increased the expression and the activity of MRP1

via a JNK-dependent pathway but not ERK and PI3K signaling pathway (Wang et al. 2015).

4.2.3 Yeast

Yeasts are unicellular eukaryotic organisms, and its biological and genetic background has been studied very clearly. Yeast has been widely used in exogenous gene expression due to its vigorous growth and short growth cycle (Valenzuela et al. 1982). The yeast system expressing foreign genes not only has the characteristics of easy culturing, rapid multiply, and easy genetic manipulation of prokaryotes but also has the functions of processing, folding, and posttranslational modification of eukaryotic proteins (Gellissen et al. 1991). In view of the unique characteristics of yeast, it is served as a recombinant expression system to produce large quantities of transporter proteins. The yeast is an ideal model organism to clone new transporter and evaluate transporter polymorphisms (Cindy et al. 2007). The mutations in the yeast genome may help to express other mammalian membrane proteins. These yeast mutant strains may be served as a powerful heterologous expression system for characterizing mammalian glucose transporters (Wieczorke et al. 2003) or dissecting the mechanisms of biosynthetic processing and intracellular transport of several proteins (Kiser et al. 2001). Although many mammal transporters have been highly expressed in yeast, there are still unresolved issues such as roles of some exogenous in the yeast and differences in posttranslational modifications between yeast and mammalian cells.

Osato et al. (2003) assayed the activity of the two non-synonymous variants of the human equilibrative nucleoside transporter (ENT1), ENT1-I216T, and ENT1-E391K using yeast expressing ENT1-I216T or ENT1-E391K. Two cytotoxic nucleoside analogs, tubercidin and 5-fluorouridine, were served as probes. The results showed that tubercidin and 5-fluorouridine were all absorbed by yeasts expressing wild type or variants. The IC_{50} values for 5-fluorouridine inhibition of growth of yeast expressing the wild-types ENT1, ENT1-I216T, and ENT1-E391K were comparable. Uptake by plasma membrane also demonstrated that variant transporters behaved similarly to wild-type ENT1 with respect to inhibition of uptake by nucleosides and nucleoside analogs. The results indicated there are no significant differences in the functional characterization between the two non-synonymous variants of ENT1 and the wild-type ENT1 (Osato et al. 2003).

Rat OAT1 (rOAT1) has been successfully expressed in the yeast *S. cerevisiae* (Sawamiphak et al. 2005). The rOAT1 contains many yeast of non-preferred codons at the N-terminus; thus, fusion of the favored codon sequence of a hemagglutinin epitope preceding the start codon may modify expression of rOAT1 in yeast. Compared with the wild-type gene, modification in the translational initiation region substantially increased rOAT1 expression. A large fraction of rOAT1 was glycosylated, showing important roles in rOAT1 targeting. rOAT1 was predominantly located in the yeast plasma membrane which confirmed correct processing. It was also found that the recombinant yeast expressing rOAT1 showed higher uptake

of para-aminohippurate and that this effect was positively related to the expression of rOAT1 (Sawamiphak et al. 2005).

Wieczorke et al. (2003) developed a new heterologous expression system for mammalian glucose transporters using a yeast *S. cerevisiae* strain. All endogenous hexose transporters are completely deleted in the *S. cerevisiae* strain. To target the heterologous glucose transporters into the yeast plasma membrane in a fully active form, additional mutations had to be introduced into the hexose transport-deficient strain. Growth tests revealed that both GLUT1 and GLUT4 are able to mediate uptake of glucose, mannose, and galactose, but not of fructose. The new heterologous expression system may be used to characterize the kinetic parameters of the human GLUT1 and GLUT4 transporters by uptake of radioactive glucose into whole yeast cells. The kinetic properties of yeast-made GLUT1 are comparable with those obtained with human erythrocyte GLUT1. In the new yeast system, the K_m of GLUT4 for glucose was determined to be about fourfold higher than that of GLUT1 (Wieczorke et al. 2003).

A yeast whole-cell system based on a yeast strain deficient in fructose uptake was developed to characterize human GLUT5 and to identify GLUT5 inhibitors (Tripp et al. 2017). The results showed that the developed yeast whole-cell system was able to uptake fructose, demonstrating activity of GLUT5. Mutations did not affect subcellular distribution of the GLUT5 protein. The K_m value of fructose uptake by GLUT5 mutants and effects (IC_{50}) of GLUT5 inhibitors on fructose uptake in the yeast whole-cell system were comparable with those in other systems including human cancer cell line (MCF-7) and GLUT5 expressed in *X. laevis* oocytes (Tripp et al. 2017).

4.3 In Situ/Ex Vivo Models

In situ/ex vivo models, such as the isolated perfused intestine, lung, liver, brain, and kidney, are often used to study drug transport. Compared to in vitro assays, in situ/ex vivo models provide a relatively more physiological setting to determinate of transporter function. Compared to in vivo models, in situ/ex vivo models avoid the effects of other organs for drug disposition.

The protocols are as follows: the perfused organ (i.e., the liver, kidney, intestine, or brain) is directly infused with a physiological buffer such as artificial blood, plasma, or saline via the major blood vessel which provide blood to the organ. The physiological buffer containing tested drug at a special concentration is continuously perfused through the organ. After a designed time, the concentration of the drug or metabolites in the organ, perfusate and physiological fluids (e.g., the bile from liver and urine from kidney), is determined (Jager et al. 2003; Mallants et al. 2005; Tian et al. 2007).

Reis et al.(2013) compared lamivudine permeability across intestine of rats by using the ex vivo *intestinal* diffusion and in situ single-pass intestinal perfusion (SPIP).The integrity of the tissue was evaluated using the markers metoprolol (high permeability) and fluorescein (low permeability). The results showed that $P_{\text{eff, SPIP}}$ was lower than $P_{\text{eff, ex vivo, } A-B}$ but larger than $P_{\text{eff, ex, vivo, } B-A}$, all of which were higher than the data from

Caco-2 cells. These results indicated that intestinal transport of lamivudine is mainly mediated by transport mechanism (Reis et al. 2013).

The in situ SPIP was used to compare permeability of PEPT1 substrate [^{3}H]-glycylsarcosine (GlySar) in the intestine of wild-type and PEPT1 knockout mice. The results showed that permeability of [^{3}H]-GlySar in the intestine was region-dependent. In wild-type mice, [^{3}H]- GlySar permeabilities were comparable in the duodenum and jejunum but were much larger than that in ileum (approximately twofold). However, in PEPT1 knockout mice, intestinal [^{3}H]-GlySar permeability was very low, and no regional permeability of [^{3}H]-GlySar was found. The results suggested that a loss of PEPT1 activity should affect the intestinal absorption of di-/tripeptides, peptidomimetics, and peptide-like drugs (Jappar et al. 2010).

Perfusion of isolated rat livers was used to study the effects of the OATP2 inhibitor rifampicin and the P-gp inhibitor quinidine on hepatic disposition of digoxin P-gp. The results showed that digoxin exposure ($AUC_{0\text{-}60min}$) in liver perfusions was significantly increased by rifampicin and decreased by quinidine. On the contrast, the exposure of digoxin metabolite was increased by quinidine and decreased by rifampicin. These results indicated that rifampicin limits the hepatic uptake of digoxin and reduced the hepatic exposure of digoxin to CYP3A by inhibiting the basolateral OATP2 function, whereas quinidine increased the hepatic exposure of digoxin to CYP3A via inhibiting the canalicular P-gp function, which demonstrated the importance of uptake and efflux transporters on hepatic drug metabolism (Lau et al. 2004). In addition, the organ can also be sliced and incubated ex vivo with drug to investigate drug transport.

4.4 In Vivo Models

In vitro models such as membrane-based assays, cell-based assays, and in situ/ex vivo models listed above are commonly used to assess the potential for interactions with uptake and efflux transporters and also used to determine basic kinetic parameters. Those assays have been successful used in identifying many substrates and inhibitors of transporters. Though the physiologically based pharmacokinetic (PBPK) modeling approaches or in vitro to in vivo extrapolation (IVIVE) techniques may be also benefited to prediction of DDIs from transporter inhibition in humans (Rostami-Hodjegan 2012), there is a limitation that the data from in vitro or in situ/ex vivo models alone may not predict exactly the effects of transporters on pharmacokinetics and the potential DDIs. In vivo transporter model is required to reflect the differential transporter function and coordination with other processes in various organs in vivo and bridge better translation to the clinic. In drug development, the transporter substrate data from preclinical in vivo studies can provide important information for understanding pharmacokinetics, tissue distribution, efficacy, and safety of drug. Transporter gene knockout models and natural mutant animal models have become useful tools in understanding the role of drug transporters and in drug disposition. With gene-editing technologies' development, animal models with gene

knockouts and modified for transporters are now commercially available. Meanwhile, a new type of in vivo model called humanized animal, carrying the corresponding human gene for replacement of a special animal transporter gene, is increasingly popular (Scheer and Wolf 2014).

4.4.1 Transporter Chemical Knockout Models

Chemical knockdown animals are common in vivo models to provide useful information on the effects of the transport in drug absorption, distribution, excretion, and metabolism. The animals (commonly mice or rats) are treated with a transporter inhibitor to become chemical knockdown model. It is critically important in selecting the specificity of an inhibitor when applying the transporter chemical knockout models to assess the role of a specific transporter. Now, there are some selective available inhibitors such as GF120918 (an inhibitor for P-gp and BCRP), LY335979 (a specific P-gp inhibitor), and Ko143 (a specific BCRP inhibitor).

In vitro, Bcrp1-mediated etoposide resistance was reversed by two structurally different BCRP/Bcrp1 inhibitors, GF120918 and Ko143, indicating that inhibition of BCRP/Bcrp1 activity might enhance antitumor activity via increasing exposure of etoposide. However, pretreatment of wild-type mice with GF120918 increased the plasma etoposide concentrations to four–five fold. Administration of GF120918 in P-gp-deficient mice did not improve etoposide oral absorption, suggesting that intestinal P-gp not Bcrp1 have a substantial effect on the oral absorption of etoposide P-gp (Allen et al. 2003).

[^{11}H]-verapamil and cyclosporine A are often selected as P-gp substrate and P-gp inhibitor, respectively, to predict the magnitude of P-gp-based drug interactions at the human BBB (Eyal et al. 2010; Sasongko et al. 2005; Muzi et al. 2009) P-gp. It was reported that cyclosporine A (mean blood concentration, 2.8 $\pm$ 0.4 μmol/L) led to an increase in ratio of [^{11}C]-verapamil AUC_{brain} to AUC_{blood} by about 88% without affecting [^{11}C]-verapamil metabolism or plasma protein binding (Sasongko et al. 2005). P-gp-based drug interactions have been demonstrated in mice and rats where P-gp has been chemically ablated with selective inhibitors of P-gp such as PSC833, GF120918, and LY335979 (Lin and Yamazaki 2003; Endres et al. 2006). It was reported that the brain/plasma ratio of verapamil in rats increased 24.1-fold following treatment with cyclosporine A (Hendrikse and Vaalburg 2002).

[^{11}C]glibenclamide positron emission tomography (PET) imaging was used to identify the role of OATP and P-gp in glibenclamide whole-body distribution, plasma kinetics, and metabolism in baboons following coadministrating OATP inhibitor rifampicin or the dual OATP/P-gp inhibitor cyclosporine A. Data from PET demonstrated the critical roles of OATP in hepatic [^{11}C]glibenclamide uptake, [^{11}C]glibenclamide metabolism, and its plasma clearance. OATP-mediated uptake also occurred in the myocardium and kidney parenchyma but not in the brain. The inhibition of P-gp did not further influence tissue distribution of [^{11}C]glibenclamide and plasma kinetics. The results demonstrated that glibenclamide distribution,

metabolism, and elimination were greatly dependent on OATP activity, especially its hepatic clearance (Tournier et al. 2013).

4.4.2 Transporter Natural Mutant Animals

Natural mutant animals are subpopulations that have a spontaneous mutation in a transporter gene, which are commonly used to characterize the special transporter functions and its contribution to the drug distribution of the substrates. For example, a subpopulation of CF-1 mice, which are deficient in P-gp in the intestinal epithelium and brain capillary endothelium, show unusual sensitivities to the avermectins, abamectin, and ivermectin, whose neurotoxicity in the mice was 100-fold higher than that in other species and mouse strains. It was consistent with deficient in P-gp that the plasma and tissue levels of ivermectin in the P-gp defect mice were markedly higher than in the insensitive mice, particularly in the brain, the target organ for toxicity (Lankas et al. 1997).

Genetic mutations of the MRP2/ABCC2 gene have been reported both in Wistar mutant TR-rats and Sprague Dawley (SD) mutant EHBR rats which exhibit hyperbilirubinemia due to impairment of the hepatobiliary excretion of conjugated bilirubin. The EHBR is a mutant rat strain with congenital conjugated hyperbilirubinemia bred from an SD rat. Transport of conjugated bilirubin, indocyanine green, and tetra bromosulfophthalein from the liver to bile is severely impaired in these rats (Jansen et al. 1985; Kurisu et al. 1991). The two animal models are invaluable for studying the mechanisms of the biliary excretory system and bile formation.

4.4.3 Transporter Genetic Knockout Models

Because the mutant animal models has limitations such as low sustainable strategy and existing compensatory, the genetic knockout models are generated by disrupting the selected transporter gene. Along with the development of gene-editing technologies, a series of genetic knockout mice relevant to drug transporters have been generated such as Mdr1a$^{-/-}$ mice (Schinkel et al. 1994), Mdr1a$^{-/-}$:Mar1b$^{-/-}$ (Schinkel et al. 1997), Bcrp1$^{-/-}$mice (Jonker et al. 2002) Mrp1$^{-/-}$ mice, Mrp2$^{-/-}$mice, Mrp3$^{-/-}$, mice Mrp4$^{-/-}$(Belinsky et al. 2005; Leggas et al. 2004; Vlaming et al. 2006; Wijnholds et al. 1997) Oatp1b2$^{-/-}$, Pept1$^{-/-}$ mice, Pept2$^{-/-}$ mice (Hu et al. 2008; Rubio-Aliaga et al. 2003), Oct1$^{-/-}$ mice, Oct2$^{-/-}$ mice and Oct1/2$^{-/-}$ mice(Jonker et al. 2001, 2003). The recombination of multiple transporter knockout models such as Mdr1a$^{-/-}$:Mar1b$^{-/-}$:Bcrp1$^{-/-}$mice, Mdr1a$^{-/-}$:Mar1b$^{-/-}$:Mrp2$^{-/-}$:Bcrp1$^{-/-}$ mice, and Mrp2$^{-/-}$:Mrp3$^{-/-}$:Bcrp1$^{-/-}$ mice have also been generated. Fortunately, most of those animal models are now commercially available

from special company. It has become an important tool to evaluate drug absorption, tissue distribution, hepatobiliary clearance, and DDIs.

Numerous case studies have proven the importance of P-gp and/or BCRP transporter in absorption, elimination, and drug tissue distribution of substrate drugs. For example, compared with wild-type mice, Mdr1a$^{-/-}$:Mdr1b$^{-/-}$ mice showed higher exposure of ivermectin in the plasma and brain following oral dose, demonstrating roles of P-gp in intestinal absorption and brain distribution of ivermectin (Geyer et al. 2009). Similarly, in Bcrp1$^{-/-}$ mice, it was reported that AUC of sulfasalazine, a BCRP substrate, was approximately 111-fold higher than that in wild-type mice after oral administration of 20 mg/kg sulfasalazine. After i.v. administration of 5 mg/kg sulfasalazine, the AUC in Bcrp1$^{-/-}$ mice was also approximately 13-fold higher than that in wild-type mice. In accordance, treatment of wild-type mice with a known inhibitor of BCRP gefitinib resulted in a 13-fold increase in the AUC of sulfasalazine compared to the AUC in vehicle-treated mice (Zaher et al. 2006).

The food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4, 5-b] pyridine (PhIP) is transported by BCRP, P-gp, and MRPs. The effects of these transporters on the pharmacokinetics of PhIP and its metabolites after oral or i.v. administration of PhIP (1 mg/kg) were investigated using two mouse strains (Bcrp1$^{-/-}$:Mdr1a/b$^{-/-}$:Mrp2$^{-/-}$ mice and Bcrp1$^{-/-}$:Mrp2$^{-/-}$:Mrp3$^{-/-}$ mice) (Vlaming et al. 2014). The results showed that compared to wild-type mice, PhIP levels in the small intestine were reduced to four–sixfold in Bcrp1$^{-/-}$:Mdr1a/b$^{-/-}$:Mrp2$^{-/-}$ and Bcrp1$^{-/-}$:Mrp2$^{-/-}$:Mrp3$^{-/-}$ mice. Fecal excretion of PhIP was reduced to 8–20-fold. Biliary PhIP excretion was reduced 41-fold in Bcrp1$^{-/-}$:Mdr1a/b$^{-/-}$:Mrp2$^{-/-}$ mice. Moreover, the carcinogenic metabolites N_2-OH-PhIP and PhIP-5-sulfate (a genotoxicity marker) were accumulated in the liver of Bcrp1; MRP2; MRP3$^{-/-}$ mice but not Bcrp1; Mdr1a/b; MRP2$^{-/-}$ mice, indicating that MRP3 is involved in the sinusoidal secretion of these toxins. The results demonstrated that Bcrp1, P-gp, MRP2, and MRP3 significantly affect tissue disposition and biliary and fecal elimination of PhIP and its carcinogenic metabolites, in turn, affecting PhIP-induced carcinogenesis (Vlaming et al. 2014).

4.4.4 Humanized Transporter Models

During early drug development, it is very important to predict human pharmacokinetics of drug or drug candidates and assess risk for DDIs from data based on preclinical in vivo animal model (Chu et al. 2013). However significant differences between animal and human in the substrate specificity, tissue distribution, and relative abundance of transporters often limit the prediction in human (Lin 1995; Martignoni et al. 2006).

The humanized mouse models seem to overcome significant differences between animal and human. There are two approaches in generating humanized mouse models: one is to introduce the human genes into the mouse genome to generate genetically humanized mouse models; and the other is to transplant the human cells

into competent recipients resulting in tissue (such as liver) humanized mouse models. These humanized transporter mice models could overcome limitations stemming from species differences in transporter expression and substrate specificity. Up to now, a series of drug transporter humanized mouse models have been described such as human OATP1A2 (hOATP1A2), hOATP1B1, and hOATO1B3 (van de Steeg et al. 2009, 2012, 2013; Salphati et al. 2014), P-gp (Sadiq et al. 2015), MRP2 (Scheer et al. 2012), BCRP (Dallas et al. 2016), and PEPT1 (Hu et al. 2014; Hu and Smith 2016).

Three transgenic mouse models with specific and functional expression of hOATP1B1, hOATP1B3, and hOATP1A2, respectively, were generated and used to investigate the role of hOATPs in the disposition of methotrexate and paclitaxel (van de Steeg et al. 2013). The results showed that compared with Slco1a/1b$^{-/-}$ mice, the humanized OATP1B1, OATP1B3, and OATP1A2 decreased plasma levels of methotrexate and increased liver and small intestinal accumulation of methotrexate and 7-hydroxymethotrexate. The humanized OATP1B3 and OATP1A2 not OATP1B1 also increased liver and small intestinal accumulation of paclitaxel (van de Steeg et al. 2013). Moreover, humanized OATP1B1 or OATP1B3 and OATP1A2 attenuated the increases in plasma levels of bilirubin monoglucuronide and bilirubin diglucuronide in mice by Slco1a/1b deficiency (van de Steeg et al. 2012).

The humanized OATP1B1 or OATP1B3 transgenic mice were used to study the roles of human OATPs in the hepatic uptake of pravastatin, atorvastatin, and simvastatin. The results showed that humanized OATP1B1 or OATP1B3 could partially restore alterations in clearance, distribution volume, and bioavailability by Slco1a/1b deficiency. Based on the animal data and human/humanized mouse liver relative protein expression factor, the fractional contributions of human OATP1B1 to oral systemic drug clearance are estimated to be 0.50, 0.46, and 0.85 for pravastatin, atorvastatin, and simvastatin, respectively. The fraction of hepatic uptake mediated by human OATP1B1 is estimated to be 0.77 for pravastatin and 0.31 for atorvastatin, all of which is within 1.5-fold of estimates based on human pharmacokinetics (Higgins et al. 2014). However, another study showed that neither the humanized OATP1B3 nor the humanized OATP1A2 significantly alter the liver or plasma concentrations of rosuvastatin and pitavastatin in Slco1a/1b$^{-/-}$ mice. Therefore, further studies are required to define its potential use and limitations of humanized mice model in the predicted drug disposition and DDIs in humans (Salphati et al. 2014).

PEPT1 is a high-capacity low-affinity transporter which is responsible for absorption of digested di-/tripeptides and some therapeutic agents (such as the β-lactam aminocephalosporins and antiviral prodrugs). However, species differences of PEPT1-mediated intestinal absorption and pharmacokinetics make it more difficult to predict systemic drug exposure in human from animals. A humanized PEPT1 transgenic mouse model has been established and evaluated using its substrates cefadroxil and [^{3}H]-GlySar (Hu et al. 2014; Hu and Smith 2016). The results showed that the humanized PEPT1 reversed the decreases in intestinal P_{eff} values of [^{3}H] cefadroxil and [^{3}H]-GlySar by PEPT1 deficiency. Compared with wild-type mice,

the humanized PEPT1 transgenic mice showed a twofold higher affinity for jejunal PEPT1 to cefadroxil and GlySar, but their maximal flux values of cefadroxil and GlySar were six–seven-fold lower, demonstrating existence of great species difference in the transport kinetics of intestinal PEPT1. More importantly, following oral administration, the plasma exposure of cefadroxil was significantly lower in the humanized PEPT1 transgenic mice than that in wild-type mice, but pharmacokinetic behaviors of GlySar were comparable in the two strains (Hu et al. 2014). Moreover, in the humanized PEPT1 transgenic mice but not wild-type mice, the relationships of AUC and C_{max} versus dose were nonlinear, which were similar to data observed from human subjects. These results suggested that the humanized PEPT1 transgenic mice may provide a valuable tool in the drug discovery process by better predicting the oral pharmacokinetic profiles of PEPT1 substrates in humans (Hu et al. 2014; Hu and Smith 2016).

BCRP is expressed in various tissues, such as the gut, liver, kidney and brain, showing the role of the transporter in limiting intestinal absorption and brain penetration of substrate compounds. A humanized BCRP transgenic mice has been generated and extensively characterized using several BCRP substrates including sulfasalazine, daidzein, genistein, rosuvastatin, and topotecan (Dallas et al. 2016). The results showed that compared with wild-type mice, following intravenous or oral administration of sulfasalazine, blood exposure of sulfasalazine in Bcrp1$^{-/-}$ mice was significantly increased to 8.3-fold (intravenous) or 117-fold (oral). Blood exposure of sulfasalazine in the humanized BCRP transgenic mice was intermediate between those observed in wild-type mice and Bcrp1$^{-/-}$ mice. Coadministration of BCRP inhibitor Ko143 remarkably increased the blood concentrations, AUC, and C_{max} of sulfasalazine in wild-type and the humanized BCRP transgenic mice but not Bcrp1$^{-/-}$ mice. Ko143 also significantly increased the brain-to-blood concentration ratio of tariquidar in wild-type mice and the humanized BCRP transgenic mice, which was in an agreement with the expression level of the brain, Bcrp1/BCRP (Dallas et al. 2016).

4.4.5 Limitations of In Vivo Animal Models

Transporter genetic knockout, spontaneous mutation, and humanized animal models have been widely employed to understand the involvement of transporters in ADME and DDIs. However, it is important to know whether the drug has similar behavior on the transporter-mediated pathways and other pharmacokinetic properties between humans and the knockout/humanized species. Meanwhile, species differences should be taken into consideration, for species differences may exist in the localization, expression, affinity, and sensitivity to inhibitors between rodent and human transporters. The other general consideration in the use of transporter knockout/humanized animals is the potential alteration of other transporters and drug-metabolizing enzymes, and the experiment data from compensatory changes in

knockout/humanized animal drug transporter models will confuse the interpretation of drug transporter and DDIs.

4.5 Imaging of Drug Transporters

Traditional method in transporter study in vitro and in vivo mentioned above is not suitable to investigate the role of transporters in the real-time drug concentrations in tissues, especially human tissues. Imaging techniques are expected to overcome those deficiencies for its non-invasive and real-time analysis at the molecular level in vivo methods.

4.5.1 In Vitro Imaging

For in vitro imaging of transporters, confocal or regular fluorescent microscopy has always been used to monitor the distribution of the fluorescent transporter substrate at the subcellular levels. Confocal laser scanning microscopy, in combination with fluorescent probes including fluorescence-labeled antibodies, enables us to obtain optical sectioning images of living or fixed cells and tissues. It provides information on the position distribution of not only the transporter proteins themselves but also the molecules or drug they transport (Tanaka and Shigenobu 1996).

P-gp function in L1210/VCR cell lines was investigated using confocal microscopy (Orlický et al. 2004). The results showed that different from sensitive cells, the L1210/VCR cell lines were not loaded with fluo-3/AM, but the presence of cyclosporine A or verapamil caused an extensive staining of L1210/VCR cells, indicating that fluo-3/AM may be used for measurement of P-gp transport activity (Orlický et al. 2004). Weaver et al. (1991) determined the intracellular distribution of fluorescent probes rhodamine 123, daunorubicin, and doxorubicin in cells expressing P-gp using the laser scanning microscopy and confocal microscopy. The results showed that rhodamine 123, daunorubicin, and doxorubicin are localized to both plasma membrane and intracellular compartments (such as cytoplasm and intracellular membrane systems). Verapamil did not alter the relative distribution of doxorubicin and daunorubicin between the cell surface and intracellular structures. But verapamil showed a trend to re-localize rhodamine 123 to intracellular sites from predominantly plasma membrane sites, indicating that characteristic of this dye differs from other two probes (Weaver et al. 1991).

Nakanishi et al. (2012) quantitatively evaluated drug-MRP2 interactions in rat SCH hepatocytes using a microscope-based fluorescence image. The drug-MRP2 interactions were assessed by determining the fluorescence change of CDF in bile canaliculi of rat SCH cells in the presence or absence of MRP2 inhibitors. Estradiol and bilirubin were served as model compounds. The two compounds are not MRP2 substrates, but in the liver, they are metabolized to estradiol-17β-glucuronide and

bilirubin glucuronides, respectively. The two metabolites are MRP2 substrates and inhibitors. The results showed that pre-exposure with estradiol significantly decreased fluorescence of CDF in the bile canaliculi of rat SCH cells and the extent of the decrease was dependent on the time of pre-exposure and the concentration of estradiol, which was associated with intracellular concentration of estradiol-17-β-glucuronide. Similarly, pre-exposure to bilirubin significantly decreased fluorescence of CDF in bile canaliculi of the rat SCH cells (Nakanishi et al. 2011; Nakanishi et al. 2012).

4.5.2 In Vivo Imaging

For in vivo imaging of transporters or evaluating transporter-mediated DDIs, the main imaging tools are PET, single-photon emission computed tomography (SPECT), and a gadolinium complex for magnetic resonance imaging (MRI). The use of non-invasive in vivo imaging techniques has the potential to contribute to a better understanding of drug transporter in tissues and the mechanisms involved in their elimination and to highlight possible effects due to DDIs. Imaging probes make it possible to investigate the transporters involved in the disposition or evaluate transporter DDIs of drug and assess transporter function and expression in vivo.

4.5.2.1 PET

PET imaging as a powerful tool could determine drug tissue concentrations noninvasively in animals and humans to assess the influence of transporters on drug tissue distribution (Wagner and Langer 2011; Langer and Müller 2004). PET could visualize the distribution of a positron (β^+)-labeled ligand (PET probes) in vivo. Isotopes for PET are positron-emitting including ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{64}Cu, and ^{68}Ga. Their radioactive half-lives are 20.4, 9.9, 2.0, 110, 762, and 68 min, respectively (Delacroix et al. 2002). A series of special probes, such as [^{11}C]-colchicine, [^{11}C]-daunorubicin, [^{18}F]-paclitaxel, [^{11}C]-verapamil, R-[^{11}C]-verapamil, (S)-[^{11}C]-carazolol, (S)-[^{18}F]-1′-fluorocarazolol, [^{11}C]-carvedilol, and [^{11}C]-loperamide, were characterized for assessing in vivo P-gp function (Elsinga et al. 2004).

R-[^{11}C]-verapamil and [^{11}C]-verapamil have been widely used for analyzing P-gp at BBB. In general, a standard 1-tissue 2-rate-constant (1T2k) or 2-tissue 4-rate-constant (2T4k) compartment model (Fig. 4.1) was used to fit to the [^{11}C]-verapamil time-activity curves in brain (Muzi et al. 2009).

For 2T4k compartmental model, the kinetic equations of radiotracer amount in tissue 1 and tissue 2 corresponding compartments are illustrated as follows.

$$dA_1/dt = K_1 \times C_p - k_2 \times A_1 - k_3 \times A_1 + k_4 \times A_2 \tag{4.2}$$

and

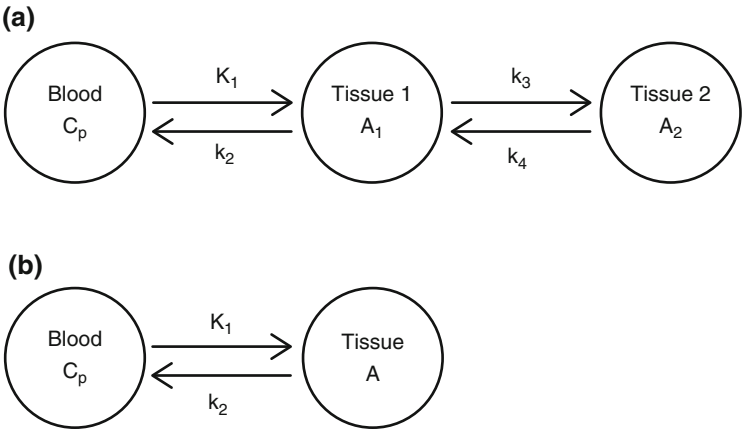

Fig. 4.1 Kinetic model for verapamil distribution in the brain. (**a**) 2T4k compartmental model. C_p is concentration of radiotracer in arterial blood; A_1 and A_2 denote the radiotracer amount in the first and second brain tissue compartment. K_1(ml (g∗min)$^{-1}$) and k_2, k_3, and k_4 (min^{-1}) are first-order rate constants describing the transfer of radiotracer between the plasma, the first, and the second brain tissue compartments. Ki corresponds to tissue uptake clearance (CL_{uptake}). (**b**) 1T2k compartmental model. A is radiotracer amount in brain tissue compartment

$$dA_2/dt = k_3 \times A_1 - k_4 \times A_2 \tag{4.3}$$

Total tissue uptake(A_t) is

$$A_t = \left(A_1 + A_2 + V_B \times C_p\right) \times \rho \tag{4.4}$$

where ρ is the tissue density in grams per milliliter and V_B is the blood volume (mL/g tissue).

The total distribution volume (V_T) is calculated as

$$V_T = (1 + k_3/k_4) \times K_1/k_2 \tag{4.5}$$

where V_T corresponds to the brain to plasma concentration ratio of the radiotracer at steady state.

For 1T2k compartmental model, the radiotracer amount in tissue is

$$dA/dt = K_1 \times C_p - k_2 \times A \tag{4.6}$$

and V_T was calculated by K_1/k_2.

It was assumed that in initial phase, amount of metabolite of 11C-verapamil was considered to be low, and the initial brain uptake rate of [^{11}C]-verapamil can be also described by the equation

$$\frac{A_t}{C_{t,p}} = (1 - V_E) \times CL_{uptake} \times \frac{AUC_t}{C_{t,p}} + V_E \quad (4.7)$$

where A_t, C_t, $C_{t,p}$, AUC_t, and V_E are the brain drug amount, blood drug concentration at time t, the area under the plasma concentration curve from 0 to t, and the initial distribution volume in the brain at time 0, respectively. V_E and CL_{uptake} may be obtained from plot of $A_t/C_{t,p}$ versus $AUC_t/C_{t,p}$. Generally, the points of the initial 20 s were excluded, and the data between 20 s and 3 min were used for the analysis.

Wagner et al. (2009) assessed P-gp function at the human BBB via comparing distribution parameters of (*R*)–[^{11}C]-verapamil in the human brain before and after administration of tariquidar. Radiotracer amount of (*R*)–[^{11}C]-verapamil was measured using PET. The result showed that coadministration of tariquidar significantly increased brain radioactivity, inducing increases in V_T and K_1 by 25% and 49%, respectively, compared with without tariquidar (Wagner et al. 2009). A strong correlation was observed between the change in brain V_T after administration of tariquidar and tariquidar exposure in plasma (Wagner et al. 2009). Similarly, coadministration of cyclosporine A also significantly increased V_T and K_1 of [^{11}C]-verapamil in the brain (Muzi et al. 2009). Age-dependent P-gp function at the human BBB was measured in seven young (mean age, 27 years) and six elderly (mean age, 69 years) healthy volunteers using PET and P-gp substrate (*R*)–[^{11}C]-verapamil. It was found that compared with young subject, the V_T values of (*R*)–[^{11}C]-verapamil in amygdala, insula, and cerebellum of elderly subjects were significantly higher by 30%, 26%, and 25%, respectively. Its efflux rate constant k_2 appeared to be negatively correlated with age. These results suggested a decrease in cerebral P-gp function with increasing age (Bauer et al. 2009). Another report also showed that although the V_T of (R)-[11C]-verapamil in whole-brain gray matter was not significantly different between the elderly (mean age 68 years) and young (mean age, 26 ± 1), after partial P-gp using tariquidar (3 mg/kg), V_T values were significantly higher in the elderly than in the young. The percentage increase in (*R*)-[$_{11}$C]-verapamil V_T by partial ABCB1 inhibition was significantly greater in elderly (40%) than in young (2%) volunteers, although tariquidar plasma concentrations were not significantly different between the young and elderly, demonstrating an increased risk for ABCB1-mediated DDIs at the BBB in elderly persons(Bauer et al. 2017). The P-gp functional differences at the BBB between the haplotypes (1236TT, 2677TT, 3435TT vs. 1236CC, 2677GG, 3435CC) of the MDR1 gene were also investigated by analyzing [^{11}C]-verapamil with the first 3-min data. It was found that distribution parameters of [^{11}C]-verapamil in the cerebral cortex, such as C_{max}, T_{max}, and AUC_{3min}, D_T and brain uptake clearance (CL_{uptake}) of [^{11}C]-verapamil, were not different between the haplotypes (Takano et al. 2006). These data did not support the assumption that the function of P-gp at the BBB is different between the haplotypes (C1236T, G2677T, and C3435T) of MDR1 gene.

The P-gp function in solid tumors both in tumor-bearing nude rats was investigated using PET method (Hendrikse and Vaalburg 2002; Hendrikse et al. 1999). [^{11}C]-verapamil and [^{11}C]daunorubicin were served as specific P-gp substrates. Tumor-bearing nude rats were developed via injecting human small cell lung

carcinoma cells (GLC4) or GLC4 expressing P-gp(GLC4/P-gp). The results showed that the [^{11}C]-verapamil content and [^{11}C]daunorubicin following administration were 184% and 159% higher in GLC4 tumors than in GLC4/P-gp tumors, respectively. Coadministration of cyclosporine A (50 mg/kg) increased [^{11}C]-verapamil content and [^{11}C]daunorubicin content in the GLC4/P-gp tumor to the levels of those in the GLC4 tumor (Hendrikse and Vaalburg 2002). Cyclosporine A also significantly increased brain distribution of [^{11}C]-verapamil by about 13-fold (Hendrikse et al. 1999). Distribution of [^{11}C]-verapamil in cancer patients was also investigated. It was found that following intravenous dose of [^{11}C]-verapamil, [^{11}C]-verapamil accumulation in the lungs, heart, and tumor of cancer patients were about 43:0%, 1:3%, and 0:9% of the total injected body dose. The mean pharmacokinetics $t_{1/2}$ in the lung, heart, and tumor were 46.2 min, 73.8 min, and 23.7 min, respectively, suggesting relatively rapid [^{11}C]-verapamil transport out of tumor tissues (Hendrikse and Vaalburg 2002).

In a PET study of mice, it was found that co-injection of GF120918 dose-dependently increased radioactivities of [^{11}C]gefitinib at 30 min following injection. Co-injection of cyclosporine A (50 mg/kg) and also induced an increase in the brain uptake of [^{11}C]gefitinib. The radioactivity level in the brain of ABCB1a$^{-/-}$:ABCB1b$^{-/-}$:Abcg2$^{-/-}$ mice at 60 min after injection was approximately eightfold higher than that in wild-type mice. These results demonstrated that both Bcrp1 and P-gp mediated transport of [^{11}C]gefitinib at the murine BBB (Kawamura et al. 2009).

(15R)-16-m-[^{11}C]tolyl-17,18,19,20-tetranorisocarbacyclin methyl ester (15R-[^{11}C] TIC-Me) was used for characterizing hepatobiliary transport. 15R-[^{11}C]TIC-Me was rapidly converted to its acid form 15R-[^{11}C]TIC within 10 s. 15R-[^{11}C]TIC was further converted to at least three metabolites, and two of the metabolites were shown to be MRP2 substrate. Because only 15*R*-[^{11}C]TIC was detected even as early as 10 s after administration, tissue uptake clearance with regard to total radioactivity could be regarded as being caused by 15*R*-[^{11}C]TIC. Data from PET scans showed that 15R-[^{11}C]TIC was localized mainly in the liver within 2 min of injection. The kinetic parameters were estimated using data within first 2 min after injection. The result showed that the intrinsic biliary clearance ($CL_{int,bile}$) of the total radioactivity in EHBR rats was only 14% of normal rats. Compared with normal rats, EHBR rats showed low liver uptake clearance, although no significance was found. The $CL_{int,bile}$ values of metabolites 3 and 2 in EHBR were, respectively, decreased to 12% and 54% of normal rats (Takashima et al. 2010). [^{11}C]-SC-62807, a main metabolite of celecoxib, was also a substrate of mouse BCRP and human BCRP and may be used for in vivo assessing function of hepatic and renal BCRP. After intravenous injection of ^{11}C-SC-62807 to wild-type and BCRP$^{-/-}$ mice, its radioactivity was measured using serial abdominal PET. The results showed that, compared with wild-type mice, both biliary and urinary excretions of radioactivity in BCRP$^{-/-}$mice was markedly decreased, accompanied by greater systemic exposure. The estimated $CL_{int,\ bile,\ liver}$ and $CL_{int,\ urine,\ kidney}$ were only 26% and 0.9% of wild-type mice, respectively (Takashima et al. 2013).

[^{11}C]-metformin was used to evaluate the function of multidrug and toxin extrusion proteins (MATEs; SLC47A) by PET. It was found that coadministration of

pyrimethamine (5 mg/kg) significantly inhibited the efflux of ^{11}C-metformin from the liver, leading to increase in ratio of liver-to-blood concentration by 2.3-fold and decrease in renal clearance by 68%, which demonstrate possibility to investigate MATE function and DDIs in the liver and kidney via measuring radioactivity of [^{11}C]-metformin using PET (Shingaki et al. 2015).

4.5.3 In Vivo Other Imaging

SPECT have been used for investigating transporter-drug interaction using a single gamma ray from isotopes. The SPECT isotopes include ^{99m}Tc, ^{111}I, ^{123}I, and ^{67}Ga. Their half-lives are 360, 168, 792, and 4320 min, respectively (Delacroix et al. 2002).

Mebrofenin labeled with ^{99m}Tc (^{99m}Tc-mebrofenin) is commonly used to investigate hepatic function and dysfunction using SPECT. After intravenous injection, ^{99m}Tc-mebrofenin is taken up by hepatocytes via OATP1 expressed in hepatic sinusoids, then transported into bile by MRP2, and finally enters the small intestine via the common bile duct. Animal experiment (Neyt et al. 2013) showed that compared with wild-type mice, OATP1a/OATP1b$^{-/-}$ mice showed significantly lower liver AUC and higher blood AUC following intravenous dose. No activity was detected in the gallbladder and intestines. Most of ^{99m}Tc-mebrofenin activity was located in the blood and urinary bladder, which was contrast to wild-type mice, in which most of the ^{99m}Tc-mebrofenin was found in the gallbladder and intestines. MRP2$^{-/-}$ mice had a higher ^{99m}Tc-mebrofenin AUC in liver and a lower ^{99m}Tc-mebrofenin AUC in both gallbladder and intestines (Neyt et al. 2013). Two analogs technetium-labeled diethylene triamine pentaacetic acid chenodeoxycholic acid [^{99m}Tc]-DTPA-CDCA and technetium-labeled diethylene triamine pentaacetic acid chenodeoxycholic acid [^{99}m]Tc-DTPA-CA were synthesized and evaluated in vitro and in vivo (Neyt et al. 2016). In vitro data showed that the two radiotracers were transported by OATP1B1 and OATP1B3 and MRP2. In vivo SPECT imaging of mice revealed uptake of both radiotracers in the liver, gallbladder, intestines, and urinary bladder. In the early phase, [^{99m}Tc]-DTPA-CDCA is taken up in the liver, gallbladder, and urinary bladder. [^{99m}Tc]-DTPA-CA is taken up in the liver and urinary bladder. In the late phase, the mean distribution of the radiotracers was mainly shown in the intestines and urinary bladder. Coadministration of OATP inhibitor rifampicin significantly decreased AUC and the maximal activity in the liver, gallbladder, and intestine (Neyt et al. 2016). ^{99m}Tc-N-pyridoxyl-5-methyltryptophan (^{99m}Tc-PMT) is clinically used for the hepatobiliary scintigraphy to diagnose disease of the hepatobiliary function and system. ^{99m}Tc-PMT uptake in hepatocytes is mainly mediated by OATP1B1 and OATP1B3 and then is excreted into bile canaliculi via MDR1 and MRP2. In vitro data demonstrated important roles of OATP1B1, OATP1B3, P-gp, and MRP2 in the transport of ^{99m}Tc-PMT. In vivo SPECT imaging showed that compared with SD normal rats, EHBR rats possessed higher liver AUC of ^{99m}Tc-PMT following intravenous administration of 99mTc-

PMT. Coadministration of P-gp verapamil further enhance AUC of ^{99m}Tc-PMT the in liver of EHBR rats, which demonstrated roles of hepatic P-gp and MRP2 in biliary excretion of ^{99m}Tc-PMT (Kobayashi et al. 2014). A candidate SPECT (^{67}Ga), ^{67}Ga-labeled bis(3-ethoxy-2-hydroxybenzylidene)-*N,N*-bis(2,2-dimethyl-3-amino-propyl)ethylenediamine), was synthesized and identified to be a P-gp substrate (Sharma et al. 2005). It was consistent with high P-gp expression that the P-gp-expressing tumor tissues accumulated less the ^{67}Ga complex compared with that of the parental tumor. Similarly, wild-type mice also showed lower ratio of the brain-to-blood concentration. Relative to wild-type mice, *mdr1a/1b*$^{-/-}$ mice showed tenfold more ^{67}Ga complex in brain parenchyma 5 min after injection of the complex. Additionally, the $AUC_{5-120\ min}$ of the radiotracer in brain of *mdr1a/1b*$^{-/-}$ mice was 17-fold greater than that of wild type, although the $AUC_{5-120\ min}$ of the radiotracer in the blood of *mdr1a/1b*$^{-/-}$mice was only 1.2-fold that of wild-type mice (Sharma et al. 2005)

Quantification with SPECT, however, remains of slightly lower performance than with PET, especially in small volume regions, due to marginally poorer image spatial resolution. But advances in multimodality SPECT/CT and other technologies such as algorithms for image reconstruction and sophisticated compensation techniques have now made quantitative SPECT viable in a manner similar to quantitative PET (Bailey and Willowson 2013). Although it has a lower sensitivity than PET imaging, MRI provides higher spatial resolution (up to 100 mm) and excellent soft tissue contrast.

MRI is a non-ionizing imaging technique which is widely used in clinical medicine. It forms an image by using magnetic fields and radio waves. H is the main nucleus used for MRI due to its abundance in tissues, but other nuclei, such as ^{13}C and ^{19}F, are also used to MRI (Månsson et al. 2006; Reid and Murphy 2008). The imaging capability of MRI of the abdominal organs (liver, kidneys, gastrointestinal tract, etc.) can be improved by contrast agents such as paramagnetic metal ions (e.g., gadolinium, manganese) or super paramagnetic iron oxides. Chelates of the paramagnetic ion gadolinium (Gd^{3+}) are the majority of contrast agents approved for MRI use in the clinic use. Gd-BOPTA and Gd-EOB-DTPA (gadoxetate) were approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for use in Europe in 2004, respectively.

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Chapter 5
Transporter-Mediated Drug-Drug Interactions and Their Significance

Xiaodong Liu

Abstract Drug transporters are considered to be determinants of drug disposition and effects/toxicities by affecting the absorption, distribution, and excretion of drugs. Drug transporters are generally divided into solute carrier (SLC) family and ATP binding cassette (ABC) family. Widely studied ABC family transporters include P-glycoprotein (P-GP), breast cancer resistance protein (BCRP), and multidrug resistance proteins (MRPs). SLC family transporters related to drug transport mainly include organic anion-transporting polypeptides (OATPs), organic anion transporters (OATs), organic cation transporters (OCTs), organic cation/carnitine transporters (OCTNs), peptide transporters (PEPTs), and multidrug/toxin extrusions (MATEs). These transporters are often expressed in tissues related to drug disposition, such as the small intestine, liver, and kidney, implicating intestinal absorption of drugs, uptake of drugs into hepatocytes, and renal/bile excretion of drugs. Most of therapeutic drugs are their substrates or inhibitors. When they are comedicated, serious drug-drug interactions (DDIs) may occur due to alterations in intestinal absorption, hepatic uptake, or renal/bile secretion of drugs, leading to enhancement of their activities or toxicities or therapeutic failure. This chapter will illustrate transporter-mediated DDIs (including food drug interaction) in human and their clinical significances.

Keywords Transporter-mediated drug-drug interaction · Intestinal absorption food-drug interaction · Hepatic uptake · Renal secretion · Transporter

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5.1 General Consideration

Drug transporters, belonging to plasma membrane transporters, play essential roles in the uptake of endogenous and exogenous substances into cells or efflux out of cells. Drug transporters are determinants of drug disposition and effects/toxicities via affecting the absorption, distribution, and excretion of drugs. Drug transporters are generally divided into solute carrier (SLC) family and ATP binding cassette (ABC) family. Widely studied ABC transporters include P-glycoprotein (P-GP), breast cancer resistance protein (BCRP), and multidrug resistance proteins (MRPs). These ABC transporters are often localized to the apical membrane of enterocytes, the luminal membranes of brain microvessel endothelial cells, the apical plasma membrane of choroid plexus epithelial cells, the canalicular membrane of hepatocytes, and the brush border membranes of renal tubules. Main functions of ABC transporters are to pump their substrates out of cell, limiting intracellular drug accumulation. SLC transporters related to drug transport mainly include organic anion-transporting polypeptides (OATPs), organic anion transporters (OATs), organic cation transporters (OCTs), organic cation/carnitine transporters (OCTNs), peptide transporters (PEPTs), and multidrug/toxin extrusion (MATEs). Most of SLC transporters (except for MATEs) related to drug transport primarily mediate uptake of drug into cells, although mediated-transports of drugs by SLC transporters sometime are also bi-directional. MATEs belong to efflux transporters, mediating efflux of organic cations.

Each transporter has a specific pattern of tissue expression, corresponding substrates/inhibitors, but their tissue expressions, substrates, and inhibitors often share great overlapping. Transporters are highly expressed in tissues related to drug disposition, such as the small intestine, liver, and kidney, implicating intestinal absorption of drugs, uptake of drugs into hepatocytes, and renal/bile excretion of drugs. Moreover, some blood-tissue barriers such as blood-brain barrier (BBB), blood-eye barrier, and maternal-fetal barrier also highly express corresponding drug transporters, protecting sensitive tissues from potentially toxic compounds. The characteristics of transporter expressions and substrates/inhibitors demonstrate their important roles in drug disposition and drug-drug interactions (DDIs). The transporter-mediated DDIs mainly occur in the liver, intestine, and kidney (Fig. 5.1). The intestine highly expresses P-GP and BCRP, which becomes reasons limiting bioavailability of orally administered substrates. Coadministration of these efflux transporter inhibitors results in an increased bioavailability of the victim drugs, leading to enhancement of their activities or toxicities, whereas concomitant medication with inducers of these efflux transporters reduces bioavailability of drug substrates, leading to therapeutic failure. Hepatocytes highly express some uptake transporters (such as OATP1B1, OATP1B3, OATP2B1, and OCT1), mediating uptake of the substrates (e.g., statins and metformin) into hepatocytes, where drugs are metabolized by drug metabolic enzymes. The efflux transporters such as P-GP, BCRP, MRP2, MATE1, and bile salt export pump (BSEP) are mainly localized in

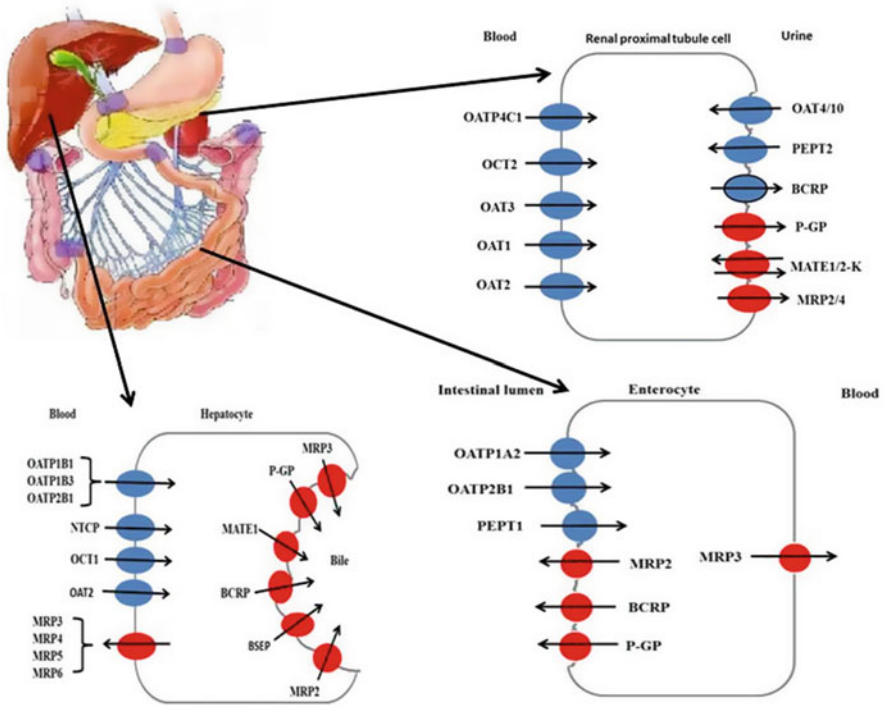

Fig. 5.1 Mainly organs/tissue and possible transporters related to DDIs. *P-GP* P-glycoprotein, *BCRP* breast cancer resistance protein, *MRPs* multidrug resistance proteins, *OATPs* organic anion-transporting polypeptides, *OATs* organic anion transporters, *OCTs* organic cation transporters, *PEPTs* peptide transporters, *MATEs* multidrug/toxin extrusions, *NTCP* sodium taurocholate cotransporting polypeptide, *BSEP* bile salt export pump

the canalicular membrane of hepatocytes, mediating excretions of drugs, their metabolites, or endogenous compounds into bile. In the kidney, secretion of cationic drugs is mainly mediated by uptake via OCT2 localized in the basolateral membrane and subsequent efflux by MATE1 and MATE2-K localized in the luminal membrane of proximal tubular cells. Inhibition of these processes by concomitantly administered drugs leads to a reduced renal clearance (CL_r) of the victim drugs.

DDI is usually assessed using a mechanistic static model recommended by the Food and Drug Administration (FDA) (http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm).

For the liver:

$$R = 1 + f_u \times I_h / K_I \tag{5.1}$$

where f_u and K_I are free fraction of inhibitor and the unbound inhibition constant (corresponding IC_{50}), respectively. I_h is referred to maximum plasma concentration

of inhibitors (C_{max}) or the estimated its maximum concentration at the inlet to the liver ($I_{inlet,max}$). $I_{inlet,max}$ is estimated as follows:

$$I_{\text{inlet,max}} = C_{\max} + k_a \times F_a \times \text{Dose}/O_h \tag{5.2}$$

where O_h is the hepatic blood flow (assumed to be 1500 mL/min/70 kg). F_a is the fraction absorbed (as default taken to be 1.0), and k_a is the absorption rate constant (as default taken to be 0.1 min^{-1}). The cutoff for R is 1.1.

For the gut lumen:

$$R = 1 + I_g/K_I \tag{5.3}$$

where I_g is referred to maximal theoretical gastrointestinal concentration of inhibitor (I_2) or maximum enterocyte concentration of inhibitor ($I_{\text{gut max}}$), which are estimated as follows:

$$I_{\text{gut,max}} = F_a \times k_a \times \text{Dose}/Q_{\text{en}} \tag{5.4}$$

$$\text{I}_2 = \text{Dose}/250\ \text{mL} \tag{5.5}$$

where Q_{en} is blood flow through enterocytes (assumed to be 300 mL/min/70 kg). The cutoff for R is set to be 11.

For the kidney, the R is estimated using the equation (Feng and Varma 2016):

$$R = \frac{1}{1 - \frac{CL_{\text{sec}}}{CL_t} \times \frac{f_u \times C_{\max}/K_I}{1 + f_u \times C_{\max}/K_I}} \tag{5.6}$$

where CL_t and CL_{sec} represent total systemic clearance and renal secretory clearance in control with no inhibitor. The cutoff for $f_u C_{\max}/K_I$ is 0.1 for OAT1/OAT3/OCT2 and 0.02 for MATEs.

Characteristics of these transporters; their expressions in tissues, substrates, and inhibitors; and their clinical significances have been reviewed in Chaps. 2 and 3; this chapter will illustrate transporter-mediated DDIs in human and their clinical significances.

5.2 P-GP-Mediated Drug-Drug Interactions and Their Clinical Significance

Numerous therapeutic drugs have been identified as P-GP substrates including cardiac glycoside digoxin, human immunodeficiency virus protease inhibitors, immunosuppressants, β-blockers, and anticancer agents. Most of P-GP substrate drugs are also substrates of cytochrome P450 3A4 (CYP3A4) and/or other drug-

metabolizing enzymes. A few P-GP substrate drugs not metabolized in humans are often served as probe drugs for P-GP function, such as digoxin, dabigatran etexilate, fexofenadine, posaconazole, ranolazine, sitagliptin, saxagliptin, talinolol, and tolvaptan. Several P-GP inhibitors such as amiodarone, atorvastatin, azithromycin, captopril, carvedilol, clarithromycin, conivaptan, cyclosporine, digoxin, diltiazem, dronedarone, erythromycin, felodipine, itraconazole, ketoconazole, lopinavir, ritonavir, quercetin, quinidine, ranolazine, ticagrelor, and verapamil are selected to investigate P-GP-mediated DDIs in human.

Typical example for P-GP-mediated DDI is digoxin. Digoxin, a typical P-GP substrate, has an oral bioavailability of 70% and is mainly eliminated via both renal and biliary secretion. DDIs of digoxin with P-GP inhibitors especially quinidine have been widely investigated. Several evidences have demonstrated that P-GP inhibitors including quinidine lead to DDIs of digoxin via increasing intestinal absorption of digoxin (Pedersen et al. 1983), decreasing biliary secretion of digoxin (Angelin et al. 1987; Hedman et al. 1990), or reducing renal secretion of digoxin (Hedman et al. 1990), which are most likely to P-GP inhibition in the small intestine, the liver, and the kidney.

In seven cardiac patients after simultaneous administration of oral digoxin and intravenous [^{3}H]-digoxin, it was found that in the presence of quinidine, plasma trough concentration of digoxin levels increased from 0.41 ± 0.25 to 0.70 ± 0.31 ng/mL, C_{max} of digoxin increased from 0.93 ± 0.34 to 1.63 ± 0.46 ng/mL, and bioavailability increased from 68.48 ± 13.35% to 79.09 ± 14.89% (Pedersen et al. 1983). In consistence, Drescher et al. (2003) reported that perfusion with quinidine reduced intestinal excretion of digoxin into the intestinal segment from 0.45% to 0.23% of the digoxin dose following intravenous digoxin using 20 cm adjacent jejunal segments isolated with the multilumen perfusion catheter in healthy subjects. Igel et al. (2007) found that perfusion with quinidine considerably increased intestinal absorption of digoxin (from 22.3 ± 8.9% to 55.8 ± 21.2% of the dose) in jejunal segment of human. Using a similar technique, it was found that considerable increases in AUC and C_{max} of digoxin were also observed in subjects coadministrated with quinidine (Igel et al. 2007). These results indicate that the increased intestinal absorption of digoxin by quinidine is due to inhibition of P-GP mediated intestinal efflux of digoxin.

Hedman et al. (1990) reported that in healthy subjects, coadministration of quinine significantly decreased both biliary and renal clearances (CL_r) of digoxin by 35% (from 134 ± 57 mL/min to 87 ± 39 mL/min) and 29% (from 155 ± 26 mL/min to 110 ± 21 mL/min), respectively. Similarly, in patients with atrial fibrillation, it was also found that coadministration of quinidine induced an average 42% reduction of the biliary clearance of digoxin (Angelin et al. 1987). In patients with atrial fibrillation, coadministration of quinidine also significantly decreased total, renal, and nonrenal clearances of digoxin following intravenous dose, inducing increased plasma digoxin levels by about twofold (Schenck-Gustafsson and Dahlqvist 1981). A report on six healthy subjects who randomly received basic treatments with 0.2 mg digoxin and coadministrations with quinidine showed that coadministration of quinidine increased digoxin levels from 0.48 ng/mL during

digoxin alone to 1.13 ng/mL during digoxin coadministrated with quinidine (Belz et al. 1982). Doering (1983) investigated effects of verapamil or verapamil plus quinidine on pharmacokinetics of digoxin. Nine healthy volunteers on basic digoxin treatment (0.125 mg t.i.d.) were treated with placebo, verapamil 80 mg t.i.d., and the combination of verapamil 80 mg and quinidine base 160 mg t.i.d. for 2 weeks in a randomized sequence. The results showed that compared to placebo, serum digoxin concentration rose by 53% from 0.62 ± 0.16 ng/mL to 0.95 ± 0.29 ng/mL during verapamil treatment and further to 1.58 ± 0.38 ng/mL (155% rise) during verapamil plus quinidine treatment (Doering 1983).

Digoxin is a drug of narrow therapeutic window, whose levels of no toxicity were reported to be <1.0 ng/mL (Mordel et al. 1993). Among 141 consecutive patients receiving digoxin, it was found that digitalis toxicity rates for digoxin alone, with amiodarone/verapamil, and with quinidine, were 4.9% (5 of 101 patients), 5.0% (1 of 20 patients), and 50% (10 of 20 patients), respectively. Toxicity rates at 1.0–2.0 ng/mL were 1 of 41 patients in digoxin alone and 4 of 15 in patients co-treated with quinidine, respectively. Toxicity rates at levels >2.0 ng/mL were 4 of 8 patients in alone and 7 of 11 patients in co-treated with quinidine, respectively. Independent relative risks (95% confidence intervals (*CI*)) of digitalis toxicity were 9.1 (95% *CI*, 2.9–13.0) for serum digoxin and 24.3 (95% *CI*, 3.4–124) for concurrent quinidine, separately, demonstrating a significant interaction between concurrent quinidine, serum digoxin of 1.0–2.0 ng/mL, and digitalis toxicity (Mordel et al. 1993).

Another clinical trial on 27 patients receiving both quinidine and digoxin showed that quinidine therapy led to increases in serum digoxin in 93% of patients (25 of the 27 patients); the digoxin levels rose from 1.4 ng/mL before quinidine to 3.2 ng/mL during quinidine. Sixteen patients (59%) during quinidine therapy developed anorexia, nausea, and/or vomiting. Three of 13 patients with only atrial arrhythmias on digoxin prior to quinidine developed new ventricular premature depolarizations after starting quinidine; 2 of these 3 as well as 4 patients with prior ventricular premature depolarizations developed new ventricular tachycardia, ventricular fibrillation, asystole, or sudden death, which demonstrated the close relationship between serum digoxin levels and the clinical course as well as alterations in ECG (Reiffel et al. 1979).

In a study using 17 patients who were kept on maintenance digoxin therapy, it was also found that 3-day quinidine treatment increased plasma digoxin levels by 20–330%. Among the 17 patients, 5 patients showed marked decreases in the renal clearance of digoxin (by about 72%). The rise in plasma digoxin levels was accompanied by symptoms suggestive of digitalis side effects in 6 out of these 17 patients. These symptoms occurred at plasma digoxin levels between 1.56 and 3.59 ng/mL and disappeared after reducing the dose of digoxin or quinidine. Four patients further developed ventricular tachycardia or ventricular fibrillation within 3 days after initiation of quinidine therapy. At that time the levels of plasma digoxin varied from 0.94 to 2.26 ng/mL by quinidine therapy (Dahlqvist et al. 1980).

Besides quinidine, other P-GP inhibitors such as ritonavir, verapamil, and talinolol also led to serious DDIs with digoxin. In a randomized, placebo-controlled

crossover study, 12 healthy male participants received oral ritonavir (300 mg twice daily) for 11 days. It was found that ritonavir significantly increased AUC of digoxin following intravenous dose by 86% and decreased nonrenal and renal clearance of digoxin by 48% and 35%, respectively (Ding et al. 2004). Another report showed that coadministration of ritonavir (200 mg twice daily) for 14 days significantly increased AUC^{0-72} of digoxin from 26.20 ± 8.67 to 31.96 ± 11.24 ng.h/mL and the AUC^{0-8} from 6.25 ± 1.8 to 8.04 ± 2.22 ng.h/mL and oral clearance decreased from 149 ± 101 mL/(h.kg) to 105 ± 57 mL/(h.kg), but renal clearance of digoxin was not affected by ritonavir (Penzak et al. 2004), indicating that ritonavir mainly affected nonrenal clearance of digoxin. Similarly, a report demonstrated that 2 weeks of pretreatment with ritonavir and saquinavir (saquinavir/ritonavir 1000/100 mg twice daily) resulted in a 1.27-fold increase in digoxin C_{max} (90% CI: 1.05–1.54) and a 1.49-fold increase in AUC^{0-72} (90% CI: 1.32–1.69), but alterations in CL_r was minor, only decrease by 12%. In line with increases in digoxin levels, triple combination of agents showed a trend to increase PR interval, indicating that the caution should be stressed when digoxin is coadministered with the three drugs (Schmitt et al. 2010). A clinical case also demonstrated that combination antiretroviral therapy led to life-threatening digoxin toxicity, especially renal impairment (Yoganathan et al. 2017).

The effect of verapamil on the pharmacokinetics of digoxin was studied in 49 chronic atrial fibrillation patients receiving a stable dose of digoxin (Klein et al. 1982). It was found treatment with verapamil (240 mg/day) significantly increased serum digoxin levels by 72%(0.76 ± 0.54 ng/mL to 1.31 ± 0.54 ng/mL during verapamil treatment). This increased effect was verapamil dose-dependent. It was also found that 7 subjects were received 160 mg verapamil then 240 mg verapamil, the serum digoxin concentrations were stepwisely increased from in from 0.60 ± 0.11 ng/mL in control to 0.84 ± 0.18 ng/mL during 160 mg verapamil and 1.24 ± 0.40 ng/mL during 240 mg verapamil, respectively. Among the 49 patients, 7 suggested signs and symptoms of digitalis toxicity (Klein et al. 1982). Interestingly, verapamil-induced suppression of renal digoxin elimination disappears following chronic verapamil exposure. A report showed that after 1 week of verapamil 240 mg/day, mean plasma digoxin significantly rose from 0.21 ± 0.01 ng/ml to 0.34 ± 0.01 ng/mL and CL_r of digoxin reduced from 197.57 ± 17.37 mL/min to 128.20 ± 10.33 mL/min. These alterations gradually subsided. After the 6-week verapamil coadministration, CL_r of digoxin normalized and plasma digoxin declined to 0.27 ± 0.02 ng/mL. The 24-h urinary recovery of digoxin increased from 46.46 ± 3.23% before to 69.78 ± 3.69% after 6 weeks of verapamil coadministration (Pedersen et al. 1982).

Oral coadministration of 100 mg talinolol was reported to significantly increase AUC^{0-72} and C_{max} of digoxin by 23% and 45%, respectively, without affecting CL_r and half-life of digoxin. Similarly, co-infusion of 30 mg talinolol with oral digoxin had no significant effects on digoxin pharmacokinetics (Westphal et al. 2000a), indicating that talinolol mainly affects intestinal absorption of digoxin. Oral administration of 200 mg itraconazole (once a day for 3 days) increased AUC^{0-72} of

digoxin by approximately 50%, and the CL_r of digoxin decreased by about 20% (Jalava et al. 1997).

Moreover, several cases about digoxin toxicity due to possible P-GP-mediated DDIs have been reported including dronedarone (Hohnloser et al. 2014; Vallakati et al. 2013), ciprofloxacin (Moffett et al. 2013), and clarithromycin (Gooderham et al. 1999; Chan et al. 2009). Englund et al. (2004) evaluated the effects of some therapeutic drugs on serum concentrations of digoxin in 618 patients undergoing therapeutic drug monitoring. P-GP inhibitors were classified as Class I, with a known effect on digoxin kinetics, or Class II, showing inhibition in vitro but no documented effect on digoxin kinetics in humans. They found that a large proportion (47%) of the digoxin patients undergoing therapeutic drug monitoring had one or more P-GP inhibitors prescribed. Univariate and multivariate analysis demonstrated that digoxin levels increased in a stepwise fashion according to the number of coadministered P-GP inhibitors. In multivariate analysis, it was found that mean digoxin levels were 0.98, 1.18, 1.24, and 1.56 ng/mL for zero, one, two, and three P-GP inhibitors, respectively. The results were even more pronounced when we analyzed only Class I P-GP inhibitors (1.29 for one and 1.43 ng/mL for two). As coadministration of digoxin and P-GP inhibitors is common, it is important to increase awareness about P-GP interactions among prescribing clinicians. The dose of digoxin may need readjustment in patients who are concomitantly receiving P-GP inhibitors.

Another example is colchicine. Colchicine, a drug of narrow therapeutic window, is also a typical substrate of P-GP (Dahan et al. 2009) and CYP3A (Tateishi et al. 1997). Most of P-GP inhibitors are also inhibitors of CYP3A. Coadministration with these P-GP/CYP3A4 inhibitors may lead to a serious adverse effect due to DDIs. In a phase I, single-sequence, two-period drug-drug interaction trial, it was found that coadministration of cyclosporine (100 mg) increased average C_{max} and AUC of colchicine by 224% and 215%, respectively (Wason et al. 2012). Some antimicrobial agents such as macrolide antibiotics and azole antifungals are also inhibitors of P-GP and CYP3A, indicating that coadministration with colchicine may lead to serious DDIs via inhibiting both P-GP and CYP3A4. Coadministration with clarithromycin was reported to lead to C_{max} and AUC of colchicine increases by 277% and 282%, respectively. Similarly, coadministration with ketoconazole was reported to increase C_{max} and AUC of colchicine by 102% and 212%, respectively (Davis et al. 2013). Terkeltaub et al. (2011) compared DDIs of oral colchicine with several CYP3A4/P-GP inhibitors including cyclosporine, ketoconazole, ritonavir, clarithromycin, azithromycin, verapamil extended release, and diltiazem extended release in healthy subjects. They found that the geometric mean ratios (*GMRs*) of colchicine C_{max} and AUC coadministrated with CYP3A4/P-GP inhibitors to those of colchicine alone were >125% across all studies, with C_{max} involving azithromycin. Significant DDIs were present when single doses of colchicine were coadministered with most of the selected CYP3A4/P-GP inhibitors. The estimated *GMRs* of colchicine C_{max} and AUC (and 90% *CI*) were 324.17 (292.32–356.01)% and 317.48 (291.79–343.17)% for cyclosporine, 297.49 (277.65–317.33)% and 339.21 (314.64–363.78)% for

clarithromycin, 267.08 (239.71–294.45)% and 345.32 (304.35–386.29)% for ritonavir, 189.52 (176.37–202.67)% and 286.75 (265.75–307.85)% for ketoconazole, 129.72 (115.29–149.88)% and 199.29 (174.69–201.88)% for verapamil extended release, 129.03 (108.22–149.84)% and 176.67 (146.49–206.84)% for diltiazem extended release, and 111.5 (94.01–128.99)% and 143.3 (124.85–161.75)% for azithromycin, respectively. The authors recommended colchicine dose reductions of 33–66% for the treatment of acute gout and 50–75% for prophylaxis for concomitant therapy with each agent, with the exception of azithromycin (Terkeltaub et al. 2011).

In line with increases in plasma exposure of colchicine, clinical reports demonstrated that organ transplant recipients on cyclosporine, following receiving colchicine, developed rhabdomyolysis and multiple organ failure (Garrouste et al. 2012; Bouquié et al. 2011). Several clinical cases showed that coadministration of clarithromycin increased the risk of fatal colchicine toxicity including severe gastrointestinal toxicity, and rhabdomyolysis and multiorgan failure, or death (Davis et al. 2013; Hung et al. 2005; Kim et al. 2013; McKinnell and Tayek 2009; Rollot et al. 2004), especially for patients with renal insufficiency (Hung et al. 2005). Tyrosine kinase inhibitor (TKI) sunitinib was also reported to increase colchicine toxicity partly due to inhibition of P-GP (Abodunde et al. 2013).

Edoxaban, an oral direct factor Xa inhibitor, is also a P-GP substrate. A report (Mendell et al. 2013) showed DDIs of edoxaban with many cardiovascular drugs. *AUCs* of edoxaban when concomitantly administrated with quinidine, verapamil, amiodarone, and dronedarone were increased by 76.7%, 52.7%, 39.8%, and 84.5%, respectively. Other oral anticoagulants (dabigatran, rivaroxaban, and apixaban) are also P-GP substrates. These results indicate that when these cardiovascular drugs are comedicated with anticoagulants, risk of bleeding in these patients may be increased; thus dosage of these anticoagulants should be readjusted (Mendell et al. 2013). In 12 male volunteers, 6-day courses of 240 mg verapamil were reported to significantly increase the C_{max} by 2.9-fold (95% *CI*: 2.4- to 4.0-fold) and *AUC* of fexofenadine by 2.5-fold (95% *CI*: 2.0- to 3.3-fold) following a single oral dose of fexofenadine (120 mg) without affecting CL_r of fexofenadine, which was attributed to inhibition of intestinal P-GP (Yasui-Furukori et al. 2005).

Paclitaxel is also a substrate of P-GP. In six patients receiving oral paclitaxel (100 mg) coadministrated with cyclosporine A (10 mg/kg), it was found that compared with control, coadministration of cyclosporine A increased *AUC* of paclitaxel from 476 ± 254 ng.h/mL to 967 ± 779 ng.h/mL, inducing a twofold increase (Veltkamp S et al. 2007). Similarly, in 14 cancer patients who received one course of oral paclitaxel of 60 mg/m^2 with or without 15 mg/kg cyclosporine A, it was found that *AUC* and C_{max} of oral paclitaxel in combination with CsA were eightfold (from 170.78 ± 85.39 ng/mL to 1451.63 ± 768.51 ng.h/mL) and twofold (from 85.39 ng/mL to 170.78 ng/mL) higher than those after oral paclitaxel alone (Meerum Terwogt et al. 1999). The *AUC* and C_{max} of paclitaxel following oral administration of paclitaxel (120 mg/m^2) in combination with oral GF120918 (1 g) in six patients were reported to be 2792.25 ± 1426.03 ng.h/mL and 307.40 ± 145.16 ng/mL, respectively, which were consistent with findings

coadministrated with cyclosporine A (2177.44 ± 1955.43 ng.h/mL and 264.71 ± 111.01 ng/mL) (Malingré et al. 2000), indicating that the increase in oral plasma exposure of paclitaxel in combination with GF120918 is of the same magnitude as in combination with cyclosporine A.

P-GP at BBB obstacles to central nervous system (CNS) delivery of P-GP substrate drugs, affecting CNS activity of drugs. Positron emission tomography (PET) is often used to assess P-GP activity at brain of human via determining radioactivity concentration of [^{11}C]-substrate in brain tissues (Ikoma et al. 2006; Sasongko et al. 2005; Kim et al. 2014; Muzi et al. 2009). It was found that coadministration of quinidine significantly increased the brain tissue radioactivity concentration of [^{11}C]-verapamil] in human, increasing brain distributions of [^{11}C]-verapamil radioactivity (AUC_{brain}/AUC_{blood}) by about 60% (Kim et al. 2014). Similarly, coadministration of cyclosporine was also reported to increase the AUC_{brain}/AUC_{blood} of [^{11}C]-verapamil by 88% without affecting [^{11}C]-verapamil metabolism or plasma protein binding (Sasongko et al. 2005). The increases in brain distribution of P-GP substrates indicate enhancement of CNS activities/toxicities. Several reports also have showed that in healthy subjects, coadministered quinidine significantly increased the systemic exposure to loperamide by over twofold, which was also associated with a markedly decreased pupil size (Kim et al. 2014; Skarke et al. 2003). Similarly, it was reported that loperamide produced no respiratory depression when administered alone, but respiratory depression occurred when loperamide was given with quinidine (Sadeque et al. 2000).

On the contrast, some agents such as rifampin, St John's wort, and carbamazepine decrease plasma exposure of P-GP substrates via inducing expression of P-GP. In healthy volunteers, it was found that after administration of rifampin (600 mg/day for 10 days), *AUC* of oral digoxin was significantly decreased by 30%, and C_{max} of digoxin was reduced by 58%, without affecting plasma exposure of digoxin for intravenous digoxin (Greiner et al. 1999). Rifampin treatment increased intestinal P-GP content 3.5-fold, which correlated with the low *AUC* of oral digoxin (Greiner et al. 1999). It was also found that during rifampin (600 mg/day for 9 days) treatment, *AUCs* of intravenous and oral talinolol were significantly lower by 21% and 35%, respectively. Treatment with rifampin resulted in a significantly increased expression of duodenal P-GP content 4.2-fold (Westphal et al. 2000b). After 12-day administration of St John's wort (900 mg daily), oral talinolol bioavailability was decreased by 25% compared with water control. Renal and nonrenal clearance, C_{max}, and T_{max} of talinolol were not significantly altered (Schwarz et al. 2007). Similarly, a 7-day pretreatment with carbamazepine was reported to significantly decrease plasma concentrations of fexofenadine following oral dose (60 mg), inducing decreases in C_{max} and *AUC* of fexofenadine by 26% and 43% of those in subjects before carbamazepine, respectively, without changing $t_{1/2}$. Carbamazepine also significantly reduced the amount of fexofenadine excreted into the urine by 44%; the CL_r of fexofenadine was not affected by carbamazepine, indicating that carbamazepine significantly decreases oral plasma exposure, probably as a result of intestinal P-GP induction (Yamada et al. 2009).

5.3 OATP-Mediated Drug-Drug Interactions and Their Clinical Significance

A variety of therapeutic drugs have been identified as substrates for OATP1A2, OATP1B1, OATP1B3, and OATP2B1. Interestingly, these OATPs share a similar and partially overlapping substrate spectrum. The best characterized family members regarding clinical relevance are OATP1B1 and OATP1B3 mainly expressed in the liver and are both mentioned in the European Manipuri Association (EMA) guideline and the FDA draft guidance for the investigating transporter-mediated DDIs. Table 5.1 lists clinically reported significant DDIs between OATP1B1 substrates and several OATP inhibitors.

Typical examples of clinically relevant OATP-mediated DDIs are statins which occur in the liver due to affecting hepatic uptake of drugs. It should be noted that these statins are also substrates of CYP450s and other transporters. For example, atorvastatin and simvastatin are mainly metabolized by CYP3A4 and fluvastatin by

Table 5.1 Effect of several OATP inhibitors on plasma exposures of some OATP1B1 substrates

OATP inhibitors	Victim drugs	*AUC*	C_{max}	References
Cyclosporine A	Atorvastatin	7.4	6.6	Shitara (2011)
	Atorvastatin	8.7	10.7	Shitara (2011)
	Cervistatin	3. 8	5.0	Shitara (2011)
	Fluvastatin	3.6	4.0	Shitara (2011)
	Pravastatin	9.9	7.8	Hedman et al. (2004)
	Pravastatin	12.0	7–8	Park et al. (2002)
	Rosuvastatin	7.1	10.6	Simonson et al. (2004)
	Repaglinide	2.4	1.7	Kajosaari et al. (2005)
	Bosentan	2.0	1.7	Binet et al. (2000)
	Ritonavir	2.0	2.2	Brennan et al. (2013)
	Danoprevir	13.6	7.2	Brennan et al. (2013)
Rifampicin	Atorvastatin	6.1	14.0	Takehara et al. (2018)
	Fluvastatin	2.9	2.5	Takehara et al. (2018)
	Pitavastatin	2.8	3.4	Takehara et al. (2018)
	Pitavastatin	8.2	5.7	Chen et al. (2013)
	Pitavastatin	5.8	4.4	Prueksaritanont et al. (2014)
	Pravastatin	2.3	2.7	Deng et al. (2009)
	Rosuvastatin	2.4	6.7	Takehara et al. (2018)
	Rosuvastatin	5.2	9.3	Prueksaritanont et al. (2014)
Gemfibrozil	Cerivastatin	5.6	3.1	Backman et al. (2002)
	Atorvastatins	2.8	2.8	Kyrklund et al. (2001)
	Rosuvastatin	1.9	2.2	Schneck et al. (2004)
	Atorvastatin	1.4	1.0	Whitfield et al. (2011)
	Simvastatin acid	2.8	2.2	Backman et al. (2000)

Data were expressed as increased folds which were defined as ratio of parameters with inhibitors to those without inhibitors

CYP2C9. Pitavastatin, rosuvastatin, and pravastatin are little metabolized by cytochrome P450 enzymes (König et al. 2013). Other transporters such as sodium/taurocholate cotransporting polypeptide (NTCP), BCRP, P-GP, and MRP2 also mediate transport of some statins. Coadministration of OATP inhibitors may increase statin plasma concentrations with the risk of side effects such as myopathy and rhabdomyolysis (Catapano 2012; Li et al. 2015) due to inhibiting hepatic uptakes of these statins.

Cyclosporine A is a potent inhibitor of OATPs, whose K_I values for OATP1B1-mediated cerivastatin and OATP1B1 atorvastatin are 0.24 (Shitara et al. 2003) and 0.31 μM (Amundsen et al. 2010), respectively. Cyclosporine A is also inhibitor of CYP3A4, whose K_I value in human hepatic microsomes was reported to be 0.98 μM (Amundsen et al. 2012). In addition, it was found that in human hepatic microsomes, cyclosporine A did not alter the metabolic rate of cervastatine up to a concentration of 3 μM (Shitara et al. 2003). R for DDI (ratio of *AUC* with inhibitor to without inhibitor) of atorvastatin with cyclosporine A in the liver may be estimated using equation, $R = 1 + [I]/K_I$, where $[I]$ was unbound concentration of inhibitor in hepatocytes. It was reported that mean C_{max} of cyclosporine A was 1880 ng/ml (1.56 μM) in patients receiving 163 ± 44 mg of cyclosporine A. Free faction in plasma and ratio of blood to plasma concentration for cyclosporine A were set to be 0.038 and 1.56 (Amundsen et al. 2012); ratio of liver to blood cyclosporine A concentration was set to be 4.13 (Guo et al. 2013). DDIs of atorvastatin with cyclosporine A based on OATBP1B1 and CYP3A4 inhibition were estimated to 2.25 and 1.39, respectively, indicating that either only considering CYP3A4 inhibition or OATP1B1 inhibition by cyclosporine A did not explain DDI of cyclosporine A. Interplay of enzyme and transporters should be introduced. It was assumed that $AUC = \text{Dose}/(fu \times CL_{\text{int;all}})$, where f_u and $CL_{\text{int,all}}$ represented the blood unbound fraction and overall intrinsic clearance, respectively. $CL_{\text{int;all}}$ is expressed as follows (Shitara et al. 2006):

$$CL_{\text{int,all}} = \frac{CL_{\text{inf}} \times CL_{\text{int,met}}}{CL_{\text{eff}} + CL_{\text{int,met}}} \tag{5.7}$$

where CL_{inf}, CL_{eff}, and $CL_{\text{int,met}}$ represent the intrinsic clearance for hepatic uptake from blood, the efflux from hepatocytes to blood, and metabolic clearance, respectively.

Thus, ratio of *AUC* of victim drug with to without inhibitor *R* is:

$$R = \frac{(CL_{\text{eff}} + CL_{\text{int,met}}/(1 + [I]/K_{I,CYP})) \times (1 + [I]/K_{I,CYP}) \times (1 + [I]/K_{I,OATP})}{CL_{\text{eff}} + CL_{\text{int,met}}} \tag{5.8}$$

where $K_{I,CYP}$ and $K_{I,OATP}$ are inhibition constants of CYP3A4 and OATP1B1, respectively. If CL_{eff} was larger than $CL_{\text{int, met}}$, *R* became:

$$R = (1 + [I]/K_{I,CYP}) \times (1 + [I]/K_{I,OATP}) \quad (5.9)$$

Thus, R (DDI) of cyclosporine A with atorvastatin was estimated to be 3.12, which was still lower than observation due to inhibition of intestinal CYP3A4 and efflux transporters (such as P-GP and BCRP) when it is orally administrated. Interestingly, it has recently been demonstrated that this inhibition is a long-lasting effect of cyclosporine A on OATP-mediated uptake with a measurable reduction of OATP1B1 activity for at least 18 h after its removal from the uptake assay (Shitara et al. 2012). It was also found that preincubation with cyclosporine A remarkably enhanced inhibition on OATP1B1 activity, inducing increases by 22-fold (K_I: 0.014 ± 0.003 μM for preincubation versus 0.31 ± 0.22 μM for coincubation) (Amundsen et al. 2010).

The effects of cyclosporine A on disposition of other statins have been investigated. Hedman et al. (2004) reported the single-dose pharmacokinetics and short-term safety of pravastatin in children undergoing regular triple-drug immunosuppressive therapy (including cyclosporine A) after cardiac transplantation. They found that $C_{\max}$ and AUC of pravastatin was 122.2 ± 88.2 ng/mL and 264.1 ± 192.4 ng.h/mL, respectively. These values were nearly tenfold higher than the corresponding values reported in hypercholesterolemic children without immunosuppressive therapy. Pravastatin is not metabolized, indicating that this increase by cyclosporine A is likely to be attributed to the inhibition of hepatic uptake transporters. Similarly, 11 patients (about 30.2 ± 12.3 months after heart transplantation) received immunosuppressive therapy (including cyclosporine A), and 8 control subjects received a daily dose of 40 mg/day pravastatin for the first 8 days which was then reduced to 10 mg/day administered until day 29. It was found that $C_{\max}$ and AUC of pravastatin in patients were 7–8 and 12 times higher than those of control subjects, respectively (Park et al. 2002). The interaction of rosuvastatin with cyclosporine A was investigated in a study with ten stable patients who underwent heart transplantation (Simonson et al. 2004) and were taking 10 mg of rosuvastatin for 10 days. It was found that the AUC and the $C_{\max}$ in transplant recipients were 7.1- and 10.6-fold of control subjects, respectively (Simonson et al. 2004).

Cyclosporine A also induces DDIs with other drugs. Brennan et al. (2013) evaluated the effect of cyclosporine A on danoprevir pharmacokinetics in healthy volunteers using a single-dose, randomized, open-label, two-sequence, three-period, crossover study (i.e., a single oral dose of danoprevir 100 mg in combination with ritonavir 100 mg, a single oral dose of cyclosporine A 100 mg, or combination of danoprevir/ritonavir and cyclosporine A). They found that the $C_{\max}$, AUC^{∞}, and concentration (at 12 h post dose) of GMRs (90% *CI*) when danoprevir/ritonavir and cyclosporine A were coadministered versus danoprevir/ritonavir or cyclosporine A alone were as follows: 7.22 (5.42–9.62), 13.6 (11.2–16.6), and 22.5 (17.4–29.3), respectively, for danoprevir; 1.97 (1.72–2.27), 2.23 (2.07–2.42), and 2.50 (2.22–2.81), respectively, for ritonavir; and 1.42 (1.29–1.57), 3.65 (3.27–4.08), and 6.15 (5.32–7.11), respectively, for cyclosporine A. These results demonstrate that a significant DDI occurs between cyclosporine A and danoprevir/ritonavir,

leading to substantial increases in exposure to danoprevir and a lesser impact on exposure to ritonavir, indicating that coadministration of danoprevir/ritonavir with potent OATP inhibitors should be undertaken with appropriate precautions.

Oral antidiabetic drug repaglinide is a substrate of CYP2C8, CYP3A4, and OATP1B1. The interaction of repaglinide with cyclosporine A was investigated in a randomized crossover study for 12 healthy volunteers. It was found that the C_{max} and *AUC* of repaglinide coadministrated with cyclosporine A were 175% and 244% of controls, respectively. The subjects with the greatest pharmacokinetic interaction had the greatest increase in blood glucose-lowering effect (Kajosaari et al. 2005).

Rifampin is also an inhibitor of OATP1B1 and OATP1B3, although it also acts as inducer of CYP3A and some transporters. Therefore, DDIs caused by rifampin are highly dependent on time. For example, intravenous 30-min infusion rifampicin (600 mg) to healthy subjects (Lau et al. 2007) was reported to significantly increase *AUC* of atorvastatin acid, 2-hydroxy-atorvastatin acid, and 4-hydroxy-atorvastatin acid by 6.8 ± 2.4-fold, 6.8 ± 2.5-fold, and 3.9 ± 2.4-fold, respectively, due to inhibition of hepatic OATP1B1and OATP1B3. On the contrast, repeated administration of rifampin (600 mg daily for 5 days) significantly decreased *AUC* of atorvastatin by 80% due to induction of CYP3A4 (Backman et al. 2005). Pitavastatin undergoes little hepatic metabolism. A report (Chen et al. 2013) showed that co-treatment of rifampin significantly increased C_{max} and *AUC* of pitavastatin by 819.2% and 573.5%, respectively. Interestingly, the increases in plasma exposure of pitavastatin by intravenous (IV) rifampicin were larger than those by oral (PO) administration of rifampicin (*AUC*, 7.6-fold increase for IV versus 5.8-fold increase for PO; C_{max}, sixfold increase for IV versus 4.4-fold increase for PO), indicating minor roles of intestinal BCRP and P-GP in the disposition of pitavastatin (Prueksaritanont et al. 2014). Unlikely to pitavastatin, either intravenous administration or oral administration of rifampicin had a greater impact on C_{max} (5.5- to 9.3-fold) than *AUC* (3.3- to 5.2-fold) of rosuvastatin, suggesting that the impact of rifampicin on rosuvastatin is primarily at the presystemic level and may be involved in inhibition of intestinal efflux transporters (such as BCRP, P-GP, and MRP2) (Prueksaritanont et al. 2014). Single oral administration of rifampicin (600 mg) significantly was also reported to increase C_{max} and *AUC* of pravastatin to 2.7-fold and 2.3-fold (Deng et al. 2009).

Gemfibrozil is another inhibitor of OATP1B1. Moreover, gemfibrozil also inhibits CYP2C8 (Wang et al. 2002; Ogilvie et al. 2006), CYP2C19, and CYP2C9 (Wen et al. 2001). In human, gemfibrozil is metabolized to gemfibrozil glucuronide, accounting for 10–15% of gembrozil in plasma (Luo et al. 2017). In addition to inhibition of OATP1B1, gemfibrozil glucuronide is also a potential mechanism-based inhibitor of CYP2C8 (Ogilvie et al. 2006). Moreover, the inhibitory effects of gembrozil glucuronide on OATP1B1 and CYP2C8 were reported to be stronger than gemfibrozil. The IC_{50} values of gemfibrozil on OATP1B1-mediated cerivastatin uptake (72.4 μM) and CYP2C8-mediated cerivastatin metabolism (28 μM) were higher than those of gemfibrozil glucuronide (24.3 μM for OATP1B1 inhibition and 4.07 μM for CYP2C8 inhibition). In six male volunteers, it was reported that plasma concentrations of gemfibrozil and gemfibrozil glucuronide at 1.5 h after the sixth dose of gemfibrozil (1200 mg/day, twice a

day) were 80 μM and 8 μM (Luo et al. 2017), respectively. These results indicate that clinically relevant to DDIs by gemfibrozil should be attributed to inhibition of OATP1B1 and these CYP450s by both gemfibrozil and gemfibrozil glucuronide.

DDIs of gemfibrozil with statins have been widely investigated. The typical example is cerivastatin. In a randomized, double-blind crossover study with ten healthy volunteers, it was found that repeated dose of gemfibrozil (600 mg twice daily for 3 days) significantly increased plasma concentrations of cerivastatin; relative alterations in AUC and C_{max} of cerivastatin were 559% and 307% of control values (Backman et al. 2002), respectively. Gemfibrozil increased the AUC cerivastatin lactone and metabolite desmethylcerivastatin (M-1) to 440% and 435% of the control values, respectively, whereas AUC of most important hydroxy metabolite (M-23) was decreased to 22% of control (Backman et al. 2002). M-1 formation was reported to be equally catalyzed by CYP2C8 and CYP3A4, and M-23 formation was predominantly mediated via CYP2C8. Decreases in the formation of M-23 by gemfibrozil administration were thought to be inhibition on CYP2C8 (Wang et al. 2002). The increases in plasma exposure of cerivastatin coadministrated with gemfibrozil may partly explain the high incidence of myopathy observed with this combination (Alexandridis et al. 2000; Marsà et al. 2002).

Repeated administration of gemfibrozil (1200 mg/day for 3 days) was reported to increase AUC of pravastatin to 202% of control. Although the CL_r of pravastatin was reduced from 25 L/h to 14 L/h by gemfibrozil, but the cumulative excretion of pravastatin into urine did not change significantly. The alteration in CL_r did not explain the increases in AUC of pravastatin by gemfibrozil (Kyrklund et al. 2003). In vitro data demonstrated that gemfibrozil and gemfibrozil glucuronide inhibited the uptake of [^{14}C]-pravastatin by human hepatocytes with K_I values of 31.7 μM and 15.7 μM. Considering the plasma concentrations of gemfibrozil and gemfibrozil glucuronide in humans, the inhibition of OATP1B1-mediated hepatic uptake of pravastatin by both gemfibrozil and gemfibrozil glucuronide would commonly contribute to the elevation of plasma concentration of pravastatin by the concomitant use of gemfibrozil (Nakagomi-Hagihara et al. 2007a). Gemfibrozil also leads to DDIs with other statins (rosuvastatin, lovastatin acid and atorvastatins) in different extents (Table 5.1).

Macrolides are known to cause severe DDIs, and most cases of DDIs have been thought to the inhibition of metabolizing enzymes. Nevertheless, several studies have investigated the effect of macrolides on OATP-mediated uptake in vitro or on the disposition of OATP substrates in humans. Seithel et al. (2007) reported that in vitro pravastatin transport can be inhibited by clarithromycin or roxithromycin. Jacobson (2004) reported that in humans, clarithromycin increased the C_{max} values of the CYP3A4 and OATP1B1 substrates simvastatin and atorvastatin by 609% and 446%, respectively. It is noteworthy that, in the same study, it was demonstrated that clarithromycin also increased C_{max} of coadministered pravastatin from 18 ng/mL to 41 ng/mL and the AUC from 54 ng.h/mL to 114 ng.h/mL, suggesting that this observed interaction may be attributable to the inhibition of OATP-mediated pravastatin uptake into hepatocytes. Consistently, Li et al. (2015) compared risk of adverse events co-clarithromycin or co-azithromycin with statins not metabolized

by CYP3A4 in large healthcare databases. A population-based cohort of older adults (mean age, 74 years) were taking a statin (rosuvastatin, pravastatin, or fluvastatin) not metabolized by CYP3A4 between 2002 and 2013 co-prescribed with clarithromycin ($n = 51{,}523$) or azithromycin ($n = 52{,}518$). They found that compared with azithromycin patients, patients co-prescribed with clarithromycin and a statin not metabolized by CYP3A4 were at increased risk of hospital admission with acute kidney injury [adjusted relative risk (RR) 1.65, 95% *CI*: 1.31–2.09], admission with hyperkalemia (adjusted RR 2.17, 95% CI: 1.22–3.86), and all-cause mortality (adjusted RR 1.43, 95% *CI*: 1.15–1.76). They gave a conclusion that among older adults taking a statin not metabolized by CYP3A4, co-prescription of clarithromycin showed statistically significant increases in risk of adverse outcomes (Li et al. 2015).

Endothelin receptor antagonist bosentan is mainly metabolized by CYP2C9 and CYP3A4 and transported by OATP1B1 and OATP1B3 (Treiber et al. 2007). In vitro data showed that cyclosporine A, ketoconazole, rifampin, and sildenafil significantly decreased OATP1B1- or OATP1B3-mediated bosentan, whose IC_{50} were lower than their effective plasma concentrations in humans, which may partly explain that coadministration of OATP inhibitors increased the plasma concentrations of bosentan in humans (van Giersbergen et al. 2007; Treiber et al. 2007; Binet et al. 2000).

OATP1B1-mediated DDIs of statins seemed to be genotype-dependent. A report showed that pravastatin is administered alone versus with darunavir/ritonavir to three groups of HIV-negative healthy participants, i.e., group 1 (*SLCO1B1∗1A/∗1A*), group 2 (*SLCO1B1∗1A/∗1B or ∗1B/∗1B*), and group 3 (*SLCO1B1∗1A/∗15*; *∗1B/∗15*, or *∗1B/∗17*). Significant DDIs of C_{max} and *AUC* with darunavir/ritonavir mainly occurred in group 3 variants; compared with control, relative change in pravastatin during darunavir/ritonavir mean *AUC* was largest in *SLCO1B1* group 3 (1.8-fold), followed by group 2 (1.6-fold) and group 1 (1.1-fold). A similar pattern was observed for the mean relative change in pravastatin C_{max}, largest in group 3 (2.3-fold), followed by group 2 (1.7-fold) and group 1 (1.1-fold). However, no significance was found among groups (Aquilante et al. 2012).

Another report also demonstrated that rifampicin elevated the plasma concentration of atorvastatin depending on *SLCO1B1* genotype. Sixteen subjects with known *SLCO1B1* genotypes (6 *c.521TT*, 6 *c.521TC*, and 4 *c.521CC*) were divided into two groups (atorvastatin-placebo group and atorvastatin-rifampicin group) randomly. In this two-phase crossover study, atorvastatin (40 mg single oral dose) pharmacokinetics after coadministration of placebo and rifampicin (600 mg single oral dose) were measured. The results showed that rifampicin increased atorvastatin plasma concentration in accordance with *SLCO1B1* 521T>C genotype, while the increasing percentage of *AUC* among *c.521TT*, *c.521TC*, and *c.521CC* individuals were 833 ± 245% versus 468 ± 233% versus 330 ± 223%. Although not statistically significant, the increase percentage of C_{max} from *SLCO1B1c.521CC* group was higher than those derived from carriers of the *SLCO1B1* wild-type (He et al. 2009). Repaglinide is a substrate of CYP2C8, CYP3A4, and OATP1B1. In a randomized crossover study, 24 *SLCO1B1*-genotyped healthy volunteers were given daily doses of 1200 mg gemfibrozil, or placebo, followed by 0.25 mg of

repaglinide on day 3. The results showed that increases in *AUC* and C_{max} of repaglinide by gemfibrozil were also gene-dependent. Extents of DDIs by gemfibrozil were *SLCO1B1 c.521CC* genotype (7.0-fold for *AUC* and 2.6-fold for C_{max}) > *c.521TC* genotypes (6.1-fold for *AUC* and 2.2-fold for C_{max}) > *c.521TT* genotypes (5.5-fold for *AUC* and 1.9-fold for C_{max}) (Kalliokoski et al. 2008). A genotype-dependent DDI of cyclosporine A on the *AUC* of repaglinide was also detected. The effect of cyclosporine A on repaglinide *AUC* was 42% lower in subjects with the *SLCO1B1 521TC* genotype than in subjects with the *521TT* (reference) genotype (Kajosaari et al. 2005).

In the interpretation of clinical DDI studies, it should be kept in mind that several inhibitors of OATPs (such as cyclosporine A) are also potent inhibitors not only of drug-metabolizing enzymes or other transport proteins (MRP2, P-GP). OATP1B1 substrates (such as atorvastatin) are often substrates of drug-metabolizing enzymes and other transporters (such P-GP and BCRP). Thus, increases in *AUC* (and/or C_{max}) of OATP1B1 substrates (such as statins) by inhibitors should be contributed to overall synergistic effects of drug metabolism and transport inhibition. For example, repaglinide is a substrate of OATP1B1. Hepatic uptake of repaglinide is mediated by OATP1B1, where repaglinide is mainly metabolized by CYP2C8. Gemfibrozil and its glucuronide are inhibitors of CYP2C8 and OATP1B1. Moreover, gemfibrozil glucuronide is also a potential mechanism inhibitor. This indicates that increases in *AUC* of repaglinide by gemfibrozil are attributed to combined effects of CYP2C8 and OATP1B1 by gemfibrozil and its glucuronide. A static model combining reversible OATP1B1 inhibition and mechanism-based CYP2C8 inhibition was successfully developed to explain the increases in the *AUC* of repaglinide by gemfibrozil (Honkalammi et al. 2011).

5.4 OAT-Mediated DDIs and Their Clinical Significance

The organic anion transporters OAT1 to OAT4 belong to the SLC22 family. OAT1 is expressed in different tissues with the highest expression in the basolateral membrane of proximal tubule cells, especially in the S2 segment. OAT2 is mainly expressed in the human liver and to a lower extent also in the kidney with the same localization as the OAT1 protein. OAT3 is present in all proximal tubule segments. OAT4 is mainly expressed in the luminal membrane of proximal tubule cells. A series of drugs have been identified as substrates or inhibitors of OATs. OAT1 and OAT2 share the same substrate spectrum. The best characterized substrates of OAT1 and OAT2 include angiotensin-converting enzyme inhibitors (e.g., captopril), diuretics (e.g., bumetanide and furosemide), β-lactam antibiotics (e.g., benzylpenicillin and ceftibuten), and antivirals (e.g., ganciclovir). Both OAT1- and OAT2-mediated transports can be also inhibited by several statins, although no OAT1- or OAT2-mediated statin transport has been detected. OAT3 is able to transport rosuvastatin, pitavastatin, and pravastatin (Watanabe et al. 2011). The OAT3-mediated transport may be inhibited by several statins including atorvastatin,

fluvastatin, pravastatin, rosuvastatin, and simvastatin (König et al. 2013). The OAT4 mediates the apical transport of various anionic drugs but exhibits a relatively narrow substrate recognition spectrum compared with OAT1 and OAT3. Only a few drugs have been identified as OAT4 substrates such as bumetanide (Hasannejad et al. 2004), torasemide (Hagos et al. 2007), estrone sulfate (Takeda et al. 2002a), methotrexate (MTX) (Takeda et al. 2002b), and cephalosporins (Takeda et al. 2002a; Khamdang et al. 2003).

OATs have great potential to participate in many clinically relevant DDIs. OAT-mediated DDIs can result from both beneficial and harmful effects associated with drug administration. Favorable results of OAT-related interactions are always connected with decreasing or inhibiting OAT-mediated transport of toxic compounds; reducing the hepatotoxicity, nephrotoxicity, or neurotoxicity of certain clinically important drugs with a low therapeutic index; and consequently alleviating their side effects or increasing clinical efficiency. On the other hand, the inhibition of OATs-dependent anion transport may also cause detrimental effects, aggravating side effects and increased toxicity of therapeutics. Although OATs are present in various tissues, nowadays, OAT-mediated DDIs are focused on the kidney.

Typical examples of OATs-mediated DDIs are interaction between β-lactam antibiotics and probenecid. Probenecid is a well-characterized inhibitor of both OAT1 and OAT3. Its K_I values for human OAT1 and human OAT3 inhibition were reported to be 4.3~12.1 μM and 1.3~9.0 μM (Jung et al. 2001; Takeda et al. 2001; Hashimoto et al. 2004; Tahara et al. 2005; Maeda et al. 2014), respectively. Less inhibitory effects (Li et al. 2006; Reid et al. 2003; Horikawa et al. 2002; Enomoto et al. 2002) were reported with human MRP2, human MRP4, human OAT2, and human OAT4 (K_I of 44.6, 2300, 766, and 54.9 μM, respectively). At clinical oral doses (0.5~2 g), unbound plasma concentrations of probenecid were reported to be 3~50 μM (Emanuelsson et al. 1987), suggesting that both human OAT1 and human OAT3 are likely to be the site of drug interactions with probenecid in vivo. Nevertheless, probenecid at high doses also inhibits other transporters and some phase II drug metabolizing enzymes; cautions should be taken when interpreting in vivo DDI data with probenecid. Probenecid was initially used to decrease the renal tubular secretion of antibiotics, especially benzylpenicillin, to prolong their elimination half-life and increase serum concentrations (Overbosch et al. 1988). Because of its uricosuric properties for inhibition of renal tubular reabsorption of urate, probenecid was once served as standard of care for prevention of gout symptoms in predisposed patients (Robbins et al. 2012). However, with new therapies such as allopurinol becoming available, the importance of probenecid in the therapy of gout is declined. Nowadays, probenecid is served as a potential OAT1 or OAT3 inhibitor to investigate contributions of OAT1 or OAT3 to the disposition of the substrate drug in human (http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm080499.htm). Moreover, probenecid also provides an excellent example for OATs-mediated DDIs.

Most of β-lactam antibiotics including penicillins and cephalosporins are mainly eliminated via renal tubular secretion mediated by renal OATs. Moreover,

cephalosporins themselves inhibit organic OTA-mediated anion uptake in a different extent. K_I value of cephalothin for OAT1-mediated organic uptake is comparable to that for OAT4, indicating that cephalothin is easily taken up via OAT1 and then is rapidly secreted from the apical membrane of tubule cells via OAT4, leading to low intracellular concentrations of cephalothin, which may explain its minimal toxicity to the kidney (Takeda et al. 2002a). However, K_I values of cephaloridine, ceftriaxone, cefamandole, cefoperazone, and cefazolin for OAT4-mediated organic uptake were higher than those for OAT1, indicating that OAT4 limits the efflux of these cephalosporins, leading to the accumulation of cephalosporins and inducing nephrotoxicity (Takeda et al. 2002a). Clinical trials have demonstrated that treatment with some cephalosporins (e.g., cephaloridine) is often associated with nephrotoxicity, leading to acute proximal tubular necrosis and renal failure (Tune 1997). Coadministration of OAT inhibitors such as probenecid may increase plasma concentrations of β-lactam antibiotics, prolong their serum half-life, and reduce their nephrotoxicity. In contrast, coadministration of OAT inhibitors may increase neurotoxicity of β-lactam antibiotics due to inhibition of brain OATs.

Extents of probenecid-mediated DDIs with β-lactam antibiotics are often dependent on the renal tubular secretion of drugs. Benzylpenicillin, a substrate of OAT3, is mainly eliminated via renal tubular secretion. It was reported that probenecid at doses of 500, 750, and 1500 mg dose-dependently increased AUC of benzylpenicillin by 39%, 82%, and 227% of controls. The CL_r and cumulative amount of benzylpenicillin were decreased along with increases in dose of probenecid. The CL_r of benzylpenicillin was larger than glomerular filtration clearance (f_pGFR) in absences of probenecid, indicating that the drug undergoes renal tubular secretion. The ratio of CL_r to f_pGFR for benzylpenicillin decreased along with increasing probenecid dose, which could be explained by the inhibition of its tubular secretion by probenecid (Maeda et al. 2014). Effect of probenecid on the pharmacokinetics of cefmenoxime (1.0 g) by a 30-min intravenous infusion was investigated in ten subjects (Sennello et al. 1983). The results showed that mean C_{max} and AUC values of cefmenoxime coadministrated with probenecid were significantly increased to 127% and 178% of those without probenecid. The mean CL_r was decreased from 159 mL/min in control to 66 mL/min in subjects coadministrated with probenecid, indicating that tubular secretion is the predominant mechanism of clearance for cefmenoxime and that probenecid increases plasma exposure of cefmenoxime via inhibiting renal tubular secretion. Cefmetazole is mainly eliminated via renal tubular secretion. A report showed that probenecid (1 g orally) significantly increased concentrations of cefmetazole following intravenous cefmetazole (2 g). The AUC cefmetazole after probenecid was estimated to be 466 ± 27 μg.h/mL, which was 1.58-fold of that without probenecid. Correspondingly, the CL_t and CL_r were decreased by 35% and 40% of control values, respectively, inferring that cefmetazole is partially eliminated via renal tubule secretion (Ko et al. 1989). Probenecid also increased plasma concentrations of cefaclor and cephradine following oral administration by about twofold (Welling et al. 1979). The estimated AUC of cefaclor and cephradine were 2.1- and 2.4-folds of those without probenecid, respectively. Coadministration of probenecid would almost double the blood levels and prolong the duration of therapeutic levels of

cefamandole. The CL_r of cefamandole decreased from 229 mL/min per 1.73 m^2 without probenecid to 57 mL/min per 1.73 m^2 with probenecid (Griffith et al. 1977). However, probenecid did not alter the plasma nor urinary concentrations of moxalactam, indicating that moxalactam appears to be eliminated primarily by the kidney via glomerular filtration not tubular secretion (DeSante et al. 1982).

Probenecid is regularly used during treatment with cidofovir. The use of antivirals such as cidofovir, adefovir dipivoxil, tenofovir, and acyclovir is limited by their severe nephrotoxicity. Uptake of these antivirals by proximal renal tubule cells is a prerequisite for development of kidney damage (Izzedine et al. 2005; Ortiz et al. 2005; Polis et al. 1995). These antivirals are often substrates of OATs. Contributions of OAT-mediated uptake by renal tubular cells to nephrotoxicity have been demonstrated. In primary cultures of human proximal tubular cells, it was found that probenecid markedly diminished cytotoxicity of cidofovir and tenofovir (Lash et al. 2018). In Chinese Hamster ovary cells expressed human OAT1, it was found that cytotoxicity of adefovir and cidofovir was increased by over 470- and 387-fold of parental CHO cells. In consistence, intracellular concentrations of parent drugs and their metabolites were increased by over 100-folds of parental CHO cells. Probenecid decreased both OAT1-mediated uptake and cytotoxicity of the two antivirals (Ho et al. 2000). Clinical trial and animal experiment also demonstrated that cidofovir coadministrated with high doses of probenecid improved renal tolerability of the cidofovir (Lalezari et al. 1995; Lacy et al. 1998; Polis et al. 1995). In immunodeficiency virus-infected patients, it was found that approximately 90% of the intravenous cidofovir was recovered unchanged in the urine in 24 h. Mean CL_t of the drug from serum (148 mL/h/kg) approximated mean CL_r (129 mL/h/kg), which was significantly higher than the baseline creatinine clearance in the same patients (83 mL/h/kg), indicating that active tubular secretion plays a significant role in the clearance of cidofovir. Concomitant high-dose probenecid decreased both CL_t and CL_r of cidofovir following intravenous administration of cidofovir. The CL_r of cidofovir after probenecid was reduced to the level of glomerular filtration, presumably by blocking the active tubular secretion of the cidofovir (Cundy et al. 1995). Similarly, the CL_r of acyclovir was reported to be almost threefold greater than the creatinine clearance, and the CL_t of acyclovir (300 mL/min per 1.73 m^2) was almost entirely to CL_r (248 mL/min per 1.73 m^2). The CL_r/CL_t was 75%, which was near to 79% recoveries from urinary, indicating that acyclovir is mainly eliminated via renal tubular secretion. Probenecid treatment increased AUC of acyclovir by 40% of controls (Laskin et al. 1982). Correspondingly, CL_t and CL_r were decreased by 29% and 32%, respectively. The results indicate that although probenecid inhibits the elimination of acyclovir, its clinical significance is probably limited because increases in $t_{1/2}$ and plasma exposure of acyclovir by probenecid are small (Laskin et al. 1982).

In a clinical study, probenecid also affects the pharmacokinetics of other OAT substrates. For example, Chennavasin et al. (1979) investigated pharmacodynamics of the furosemide-probenecid interaction in human. The subjects intravenously received 40 mg furosemide (over 2–3 min period) with and without pretreatment with probenecid (1 g). They found that probenecid treatment significantly increased

plasma furosemide concentration. Correspondingly, probenecid treatment decreased the CL_t, CL_r, and nonrenal clearance of furosemide from 2.04 ± 0.42 mL/kg/min to 0.56 ± 0.05 mL/kg/min, from 1.04 ± 0.31 mL/kg/min to 0.29 ± 0.06 mL/kg/min, and from 1.00 ± 0.18 mL/kg/min to 0.27 ± 0.03 mL/kg/min, respectively. Similarly, coadministration of probenecid was reported to significantly increase plasma concentrations of orally administered furosemide in healthy volunteers (Vree et al. 1995). The CL_r values of frusemide and acyl glucuronide after probenecid treatment were decreased to 35% and 29% of those in subjects without probenecid, respectively. The nonrenal clearance of frusemide was also decreased to 42% of that in subjects without probenecid, indicating that DDI of frusemide with probenecid was also involved in other mechanisms including phase II metabolizing enzymes. However, reports about effect of probenecid on natriuretic action of frusemide are often contradictory. Hsieh et al. (1987) reported that in eight hospital patients treated with oral furosemide, probenecid (0.5 g twice a day) was given for 3 days and then discontinued. They found that the 24-h urinary sodium excretion significantly decreased from 56.3 ± 7.2 mmoL/day to 35.9 ± 7.1 mmoL/day when probenecid was coadministrated and then significantly increased to 61.7 ± 10.3 mmoL/day after probenecid was discontinued. Similarly, Honari et al. (1977) reported that 1-week pretreatment with probenecid decreased both urine flow and the excreted fraction of filtered sodium, despite the rising plasma furosemide concentration. In contrast, Sommers et al. (1991) reported that pretreatment with probenecid significantly increased the diuretic efficiency of frusemide following intravenous dose during the first 90-min period after frusemide administration. In eight normal volunteers, Brater (1978) also found that pretreatment with probenecid increased the overall response to furosemide by prolonging its effect. Sodium excretion in 8 h due to 40 mg of furosemide rose from 262 ± 16 mEq to 358 ± 11 mEq after probenecid, and urine volume increased from 3265 ± 275 to 4165 ± 83 mL. Analysis of the time course of the increased diuresis and natriuresis showed that probenecid actually decreased the response for the first 60–90 min after furosemide but increased the subsequent response, sufficiently resulting in a greater overall effect (Brater 1978). In consistence, Chennavasin et al. (1979) found that the initial urinary excretion rate of sodium after pretreatment with probenecid was less than that of furosemide alone, but after approximately 60 min, the response with probenecid pretreatment became significantly higher. Probenecid treatment caused a significant shift to the right of the relationship between serum concentration of furosemide and sodium excretion rate. But the relationship between urinary furosemide concentrations and sodium excretion rate was not affected by probenecid pretreatment (Chennavasin et al. 1979). In 20 healthy young men who received placebo and low (1000 mg/day) or high (2000 mg/day) doses of probenecid for 1 week, it was found that probenecid reduced serum uric acid in a dose-dependent manner but did not alter total natriuresis in 4 h. The sodium excretion rate in the first 30 min following intravenous furosemide (0.5 mg/kg) was reduced after probenecid regimens while that of later periods was increased (Walshaw et al. 1992).

Another example is the antineoplastic agent MTX. MTX is a cytotoxic drug with a narrow therapeutic index. In patients, CL_t of MTX (107.6 ± 15.5 mL/min) was

near to CL_r (103.5 ± 17.1 mL/min), indicating that MTX is mainly eliminated via renal tubular secretion. Coadministration of probenecid decreased the CL_t and CL_r of MTX to 68.7 ± 14.2 mL/min and 45.7 ± 7.5 mL/min, respectively. Correspondingly, $t_{1/2}$ and plasma concentrations of MTX were significantly increased. For example, the mean serum concentration at 24 h following administration of MTX coadminstrated with probenecid was 0.88 μM, which was significantly greater than concentration (0.20 μM) in patients who had not received probenecid (Aherne et al. 1978). Similar increases were also found in other clinical trials (Aherne et al. 1978; Lilly and Omura 1985). These increased plasma concentrations of MTX indicate increases in the risk of MTX toxicity, which was confirmed by a report that a patient with rheumatoid arthritis developed life-threatening pancytopenia resulting from low-dose oral MTX toxicity potentiated by probenecid (Basin et al. 1991). Moreover, coadministration of probenecid significantly increased concentration of MTX in cerebrospinal fluid of human (Howell et al. 1979; Bode et al. 1980), which is likely to be due to inhibition of brain OAT3.

Another OAT inhibitor is the gemfibrozil, although gemfibrozil is also inhibitor of OATP1B1 and OATP1B3. Some statins such as rosuvastatin, pitavastatin, and pravastatin were reported to be substrates of human OAT3 (Nakagomi-Hagihara et al. 2007b; Watanabe et al. 2011), indicating OAT3 contributes to the renal uptake of these statins in humans. A report also demonstrated that in vitro gemfibrozil and two of its metabolites potentially inhibited OAT3-mediated uptake of pravastatin (Nakagomi-Hagihara et al. 2007b). A report showed that coadministrated gemfibrozil increased pravastatin plasma concentrations, which was thought to be mainly attributed to inhibition of hepatic OATPs (Kyrklund et al. 2003). However gemfibrozil also decreased CL_r of pravastatin by 44%, which is likely attributed to renal OAT3 inhibition (Kyrklund et al. 2003). Coadministration with gemfibrozil also was reported to increase plasma concentrations of the dipeptidyl peptidase IV (DPP-IV) inhibitor sitagliptin (Arun et al. 2012). The elimination of sitagliptin is chiefly via OAT3-mediated renal active secretion and minimally via metabolism, indicating that the DDI with gemfibrozil mainly results from renal OAT3 inhibition.

5.5 OCT- or MATE-Mediated DDIs and Their Clinical Significance

Many basic drugs are often substrates or inhibitors of OCTs and MATEs. In the liver, OCT1 and OCT3 are mainly expressed in basolateral membrane of hepatocytes, where they mediate the uptake of their substrates from the sinusoidal blood into hepatocytes, whereas MATE1 expressed in the apical membrane is thought to export drugs into the bile (Yonezawa and Inui 2011). In renal proximal tubule cells, OCT2, MATE1, and MATE2-K are thought to be important components of the renal tubular secretory system for basic drugs (Yonezawa and Inui 2011; Motohashi and Inui 2013). The drug most intensively studied as substrate of OCTs and MATEs is

the antidiabetic metformin. Metformin is a hydrophilic base which exists at physiological pH as the cationic species (>99.9%). Consequently, its passive diffusion across cell membranes is very limited. Transport of metformin across cellular membranes is primarily mediated by transporters such as OCTs and MATEs. The apparent K_m values of metformin transport via OCTs and MATEs (Elsby et al. 2017; Kimura et al. 2005; Lechner et al. 2016; Nies et al. 2009) were reported to be 208~4565 μM for MATE1, 2275~2986 μM for MATE2-K, 1470~5422 for OCT1, 990~1608 μM for OCT2, and 2260 μM for OCT3, respectively.

Most DDIs in humans are focused on inhibition of OCTs and/or MATEs by H2-receptor antagonist cimetidine. Cimetidine is first identified as an inhibitor of OCTs, whose K_I values for OCT1 inhibition and OCT2 inhibition (Elsby et al. 2017; Ito et al. 2012a) were reported to be 101~275 μM and 95~207 μM, respectively. Compared with OCTs, cimetidine has higher affinity to MATEs, whose K_I values for MATE1 inhibition and MATE2-K inhibition (Elsby et al. 2017; Ito et al. 2012a) are 1.1~3.8 μM and 2.7~6.9 μM, respectively. Mean unbound steady-state plasma concentrations of cimetidine following oral administration of standard daily dose (1000 mg) were reported to be 2.03~5.20 μM (Somogyi and Gugler 1983), which are near to or higher than K_I values for MATE1 and MATE2-K inhibition but below K_I values for inhibition of OCTs, indicating that at therapeutic dose of cimetidine, transporter-mediated DDIs by cimetidine mainly result from MATE inhibition.

The first reported DDI of cimetidine with organic cationic drugs is interaction between cimetidine and procainamide (Somogyi and Heinzow 1982). Approximately 50% of administered procainamide is eliminated via the kidney in form of unchanged drug (Karlsson 1978). In six healthy volunteers, it was found that coadministration of cimetidine significantly increased *AUC* of procainamide by an average of 35% of that in subjects with cimetidine (from 27.0 ± 0.3 μg.h/mL to 36.5 ± 3.4 μg.h/mL). Correspondingly, the CL_r of procainamide during cimetidine treatment was reduced to 56% of that in subject without cimetidine (from 347 ± 46 mL/min to 196 ± 11 mL/min). Moreover, coadministration of cimetidine also led to increase in *AUC* of its metabolite N-acetylprocainamide by a mean of 25% and decrease in CL_r of N-acetylprocainamide by 24% (Somogyi et al. 1983), demonstrating that cimetidine increases *AUC* of procainamide mainly by inhibiting renal tubular active secretion of procainamide. The increase in plasma exposure of procainamide by cimetidine may increase risk of procainamide cardiotoxicity. In 36 hospitalized male patients receiving procainamide for the treatment of ventricular arrhythmias, it was found that a 3-day comedication with cimetidine (300 mg every 6 h) increased the average steady-state procainamide and N-acetylprocainamide concentrations by 55% and 36%, respectively. Twelve patients experienced mild to severe symptoms of procainamide toxicity, indicating that in patients, especially older patients, to prescribe this combination should be monitored carefully for adverse side effects (Bauer et al. 1990).

Interactions between cimetidine and metformin are also widely studied. In humans, metformin is eliminated mainly by renal excretion in the unchanged form. The population mean Cl_r of metformin was reported to be about 4.3-folds of creatinine clearance, which is likely to be attributed to extensive renal tubular

secretion (Graham et al. 2011). In seven healthy subjects, it was reported that coadministration of cimetidine (400 mg twice daily) remarkably increased the C_{max} and AUC^{0-24h} of metformin (250 mg once daily) by 73% (from 0.59 ± 0.24 μg/mL to 1.02 ± 0.39 μg/mL) and 46% (4.26 ± 1.64 μg.h/mL to 6.23 ± 2.09 μg.h/mL), respectively. The CL_r of metformin was decreased by 28%, but metformin amount excreted in urine over 24 h was unaltered (Somogyi et al. 1987). Several reports have demonstrated association of increases in plasma exposure of metformin by cimetidine with severe lactic acidosis (Seo et al. 2013; Dawson and Conlon 2003). Coadministration of cimetidine also has increased plasma concentrations and reduced CL_r of other cationic drugs such as the ranitidine, dofetilide, pindolol, and varenicline (Abel et al. 2000; Feng et al. 2008; Somogyi et al. 1992; van Crugten et al. 1986).

Another potent inhibitor of MATEs is the antiprotozoal pyrimethamine, whose K_I values for MATE1 and MATE2-K inhibition (Elsby et al. 2017; Ito et al. 2012b) were reported to be 0.083~0.131 μM and 0.056 μM, respectively. Although pyrimethamine also inhibits both OCT1 and OCT2, the K_I values (4.46 μM for OCT1 and 4.55 μM for OCT2, respectively) (Elsby et al. 2017) are remarkably higher than data for MATE1 and MATE2 inhibition. The free C_{max} of plasma pyrimethamine at therapeutic dose (50 mg) was reported to be 0.298 μM (Elsby et al. 2017), higher than K_I values for MATE inhibition, but below K_I values for OCT inhibition, indicating that at therapeutic dose, pyrimethamine-induced DDIs mainly result from MATE inhibition. Kusuhara et al. (2011) investigated effects of pyrimethamine on pharmacokinetics of metformin following oral administration of metformin (500 mg) to healthy subjects. They found that oral administration of pyrimethamine (50 mg) significantly increased C_{max} and AUC of metformin by 42% and 39% of control values, respectively. In consistence, the CL_r of metformin was decreased by 35%. More importantly, although pyrimethamine increased plasma exposure of metformin, antihyperglycemic activity of metformin was attenuated (Oh et al. 2016). It was reported that coadministration of pyrimethamine (50 mg) significantly induced increases in AUC and C_{max} of metformin by 1.68-folds and 1.02-folds of control values, respectively, but significantly decreased the ability of metformin to lower glucose levels. The antihyperglycemic effects of metformin were assessed using oral glucose tolerance tests (OGTTs) before and after the metformin dose. The results showed that the antihyperglycemic effects of metformin were decreased by pyrimethamine treatment. The mean differences (90% confidence interval) in mean (ΔC_{glu}) and maximum serum glucose concentrations (ΔG_{max}) and in 2-h post-OGTT serum glucose concentration were −0.6 (−1, −0.2),−0.9 (−1.6, −0.3), and −0.5 (−1.1, 0.1) mM, respectively. The phenomenon may be partly due to inhibition of the OCT-mediated hepatic uptake of metformin (Oh et al. 2016). Several reports have supported above deduction. A report showed that coadministrated verapamil impaired glucose-lowering effect of metformin, leading to decreases in ΔG_{max} and difference in the area under the glucose concentration time curve from 0 to 60 min after glucose ingestion (ΔAUC_{gluc60}) by 62.5% and 101% of those without verapamil, respectively, without altering the C_{max} and the AUC of metformin (Cho et al. 2014). Clinical report also demonstrated that the metformin-mediated decrease in

glucose is impaired in individuals expressing genetic variants of OCT1 (Shu et al. 2007). On the contrast, glucose-lowering action of metformin was enhanced by rifampin due to hepatic OCT2 induction. In 16 healthy subjects, it was found that 10-day treatment with rifampin significantly increased the difference in ΔG_{max} by 41.9% and ΔAUC_{gluc60} by 54.5%. In accordance, rifampin increased OCT1 mRNA levels 4.1-fold in peripheral blood cells, suggesting that rifampin increases OCT1 expression and hepatic uptake of metformin, leading to enhanced glucose-lowering action of metformin (Cho et al. 2011).

Trimethoprim is also a potential inhibitor of MATEs (K_I: 2.64 μM for MATE1 inhibition and 0.35 μM for MATE2 inhibition, respectively) and weaker inhibitor of OCTs (K_I: 27.7 μM for OCT1 inhibition and 137 μM for OCT2 inhibition, respectively) (Elsby et al. 2017). Free C_{max} of trimethoprim following oral administration of trimethoprim (200 mg) to human was reported to 7.84~4.26 μM (Elsby et al. 2017), indicating that trimethoprim at clinical dose possibly leads to DDI with MATE substrates. Vlasses et al. (1989) investigated the effect of trimethoprim on the disposition of procainamide (1 g) and its active metabolite N-acetylprocainamide in ten healthy men. They found that coadministration of trimethoprim (200 mg) decreased the mean CL_r of procainamide and N-acetylprocainamide by 45% and 26% compared with placebo. In accordance, coadministrated trimethoprim increased mean AUC^{0-12} values of procainamide N-acetylprocainamide by 39% and 27%, respectively. The corrected QT interval at 2 h after the procainamide dose was 0.40 ± 0.02 sec with placebo and 0.43 ± 0.03 second with trimethoprim. Similarly, in eight healthy men who received oral sustained-release procainamide (500 mg every 6 h for 3 days) alone and with oral trimethoprim (200 mg daily for 4 days), it was found that concomitant trimethoprim significantly increased AUC^{0-12} of both procainamide and N-acetylprocainamide (by 63% and 52%, respectively), with concurrent decreases in their CL_r (by 47% and 13%, respectively). There was a small but significant increase in the corrected QT interval with procainamide administration, which increased further with trimethoprim coadministration (Kosoglou et al. 1988).

Concomitant medication with trimethoprim also increases plasma exposure of metformin. In 12 healthy volunteers, it was found that C_{max} and AUC^{0-24} of metformin during trimethoprim treatment were 122.8% and 129.5% of control values, respectively. Metformin CL_r during trimethoprim treatment was reduced by 26.4%. Similar findings in coadministration of metformin with pyrimethamine (Oh et al. 2016) and coadministration of trimethoprim almost abolished glucose-lowering action of metformin (Müller et al. 2015). Moreover, the extent of the trimethoprim-metformin interaction appears to depend on genotype of MATE1 or OCT2. In volunteers polymorphic for both OCT2 and MATE1, trimethoprim had no relevant inhibitory effects on metformin kinetics (Grün et al. 2013).

Another class of drugs with potent inhibitory effects on OCTs and MATEs are TKIs. Minematsu and Giacomini (2011) investigated the inhibitory effects of seven TKIs (imatinib, dasatinib, nilotinib, gefitinib, sunitinib, lapatinib, and sorafenib) on OCT1-, OCT2-, OCT3-, MATE1-, and MATE2-K-mediated metformin uptake in HEK293 cells that expressed corresponding transporters. They found that except lapatinib and sorafenib, other five TKIs are potent inhibitors of the transporters, with

IC_{50} values generally in the low μM range. Comparison of the unbound $C_{max,sys,p}[I]$ and IC_{50} values of TKIs against human OCT1, OCT2, OCT3, MATE1, and MATE2-K showed that imatinib (for OCT1, MATE1, and MATE2K), nilotinib (for OCT3), gefitinib (for MATE2K), and erlotinib (for OCT1 and MATE2K) exerted potent inhibitory effects at clinically relevant concentrations, with $[I]/IC_{50} \geq 0.1$.

Cisplatin is a platinum-based anticancer drug, the use of which is limited by severe nephrotoxicity. In general, cisplatin is an excellent OCT2 substrate but a poor substrate of either MATE1 or MATE2-K. The in vivo roles of OCT2 in cisplatin-induced nephrotoxicity have been widely investigated (Ciarimboli et al. 2010; Filipski et al. 2009; Zhang and Zhou 2012). In cancer patients receiving cisplatin treatment, it was reported that a nonsynonymous single-nucleotide polymorphism (*rs316019*) in the *SLC22A2* gene was associated with reduced cisplatin-induced nephrotoxicity (Filipski et al. 2009). In Chinese cancer patients, it was also found that *SLC22A2* gene polymorphism *808 G/T* and cimetidine could attenuate cisplatin nephrotoxicity (Zhang and Zhou 2012). Verapamil and cimetidine were reported to reduce cisplatin-induced nephrotoxicity in human (Offerman et al. 1985; Sleijfer et al. 1987) via inhibiting OCT2-mediated cisplatin uptake. Animal experiments have shown that *Oct* gene knockout or coadministration of OCT inhibitors cimetidine, imatinib, tropisetron, and carvedilol protects rodents from cisplatin-induced nephrotoxicity (Ciarimboli et al. 2010; Franke et al. 2010; Guo et al. 2018; Tanihara et al. 2009; Zirak et al. 2014). These results indicate that OCT2-selective inhibitors may be used to attenuate nephrotoxicity of cisplatin. It should be noted that the complex interplay between OCT2 and MATE activity plays a crucial role in the nephrotoxicity of cisplatin (El-Arabey 2017; Yokoo et al. 2007). Importantly, inhibition or disruption of MATE1 were reported to enhance nephrotoxicity of cisplatin (Nakamura et al. 2010; Shen et al. 2013) due to inhibiting efflux of efflux cisplatin from renal tubular cells. For example, cimetidine is a selective inhibitor of MATE transporters at therapeutic doses due to its differential potencies for OCT2 and MATE transporters. Cimetidine can inhibit both OCT2 and MATEs in vivo at supratherapeutic doses. It was reported than in human, high doses of cimetidine could reduce cisplatin-induced nephrotoxicity as measured by effective renal plasma flow and glomerular filtration rate due to inhibition of OCT2 (Sleijfer et al. 1987; Sprowl et al. 2013), minimally affected human pharmacokinetics or antitumor activity of cisplatin (Sprowl et al. 2013). Clinical trial (Kou et al. 2018) and animal experiments (Li et al. 2013) showed that ondansetron can enhance cisplatin-induced nephrotoxicity, which is likely to be attributed to inhibition of MATEs. Nevertheless, the risk of using chemical inhibitors as a cisplatin nephroprotectant should be carefully addressed given the opposing effect of OCT2 and MATEs in cisplatin intrarenal accumulation and toxicity.

Ranolazine and metformin may be frequently coadministered in subjects with chronic angina and comorbid type 2 diabetes mellitus (T2DM). Zack et al. (2015) evaluated the pharmacokinetics and safety of metformin (1000 mg BID) when administered with ranolazine (1000 mg BID) or ranolazine (500 mg BID) in subjects with T2DM. They found that ranolazine dose-dependently increased plasma exposure of metformin at steady-states. Compared with metformin (1000 mg) alone, coadministration of ranolazine (1000 mg) with metformin resulted in 1.53- and

1.79-fold increases in steady-state C_{max} and AUC^{τ} of metformin, respectively. And coadministration of ranolazine (500 mg) with metformin resulted in 1.22- and 1.37-fold increases in C_{max} and AUC^{τ} of metformin, respectively. These results indicated that when ranolazine (1000 mg BID) is coadministrated, a dose adjustment of metformin may be required metformin (Zack et al. 2015).

5.6 Other Transporter-Mediated Drug-Drug Interactions and Their Clinical Significance

BCRP is highly expressed in the intestine, liver, and kidney, where it mediates intestinal efflux, biliary excretion, or tubular excretion of drugs. Topotecan is a typical substrate of P-GP and BCRP (Li et al. 2008). GF120918 (elacridar) is a strong inhibitor of P-GP (IC_{50}:0.38 μM) and BCRP (IC_{50}: 0.3 μM) (Ahmed-Belkacem et al. 2005; Luo et al. 2002). Single oral administration of elacridar (1 g) was reported to significantly alter pharmacokinetics of topotecan following intravenous or oral administration of topotecan (1 mg/m^2) to humans (Kruijtzer et al. 2002). For oral administration of topotecan, coadministration of elacridar significantly increased topotecan AUC and C_{max} by 1.43-fold and 1.80-fold of controls. For intravenous topotecan, oral administration of elacridar had a small but statistically significant effect on the AUC and CL_t of topotecan without affecting $t_{1/2}$. The mean C_{max} of elacridar was 0.77 μM (Malingré et al. 2001). Assuming that f_u was 0.01, thus estimated R-value ($1 + f_u \times C_{max}/IC_{50}$) was less than 1.1. These results indicate that oral administration of elacridar resulted in a significant increase of the systemic exposure of oral topotecan via inhibiting intestinal BCRP and P-GP and that inhibition of hepatic P-GP and BCRP by oral elacridar were minor, which may also explain that oral administration of elacridar had small impact on AUC of topotecan following intravenous dose.

Rosuvastatin is a substrate of BCRP, OATPs, and OATs. Fostamatinib, an orally inhibitor of spleen tyrosine kinase, is a prodrug (R788), which is completely metabolized by dephosphorylation in the enterocytes to the active metabolite R406. It was reported that oral coadministration of fostamatinib significantly increased rosuvastatin AUC by 96% and C_{max} by 88% and also increased simvastatin acid AUC by 74% and C_{max} by 83% of controls (Martin et al. 2016), respectively. Fostamatinib and R406 are strong inhibitors of BCRP with IC_{50} of 51 and 31 nM, respectively. R406 is also poor inhibitor of OATP1B1 ($IC_{50} > 10$ μM), but not inhibitor of OAT3 (Elsby et al. 2016). The mean C_{max} of R406 at steady-state plasma at fostamatinib (100 mg twice daily) was reported to be 1.86 μM (Martin et al. 2016). Free fraction of R406 was set to 0.018 (Elsby et al. 2016). Assuming that fostamatinib was completely converted to R406, $f_u \times I_{inlet,\ max}$ was estimated to be 0.241 μM. Both $f_u \times I_{inlet,max}$ and $f_u \times C_{max}$ were much less than IC_{50} for OATP1B1 inhibition. The R-values ($1 + f_u \times I_{inlet,max}/IC_{50}$ and $1 + f_u \times C_{max}/IC_{50}$) were less than 1.1, indicating inhibition of hepatic OATP1B1 by R406 is minor. But for

hepatic BCRP inhibition, the R-values $1 + f_u \times C_{max}/IC_{50}$ and $1 + f_u \times I_{inlet,\ max}/IC_{50}$ were calculated to be 2.02 and 8.8, respectively. $I_{gut,amx}$ of R 406 and I_2 of fostamatinib were estimated to be 57.6 μM and 691 μM following oral 100 mg of fostamatinib. The R-values of R406 and fostamatinib for intestinal BCRP inhibition were estimated to be 1858 and 13,820, respectively. These results indicate that DDIs of fostamatinib with statins should be mainly attributed to both intestinal BCRP and hepatic BCRP inhibitions.

Eltrombopag, a platelet-increasing agent, is also an inhibitor of both OATP1B1 and BCRP with IC_{50} of 2.7 μM. A clinical report (Allred et al. 2011) showed that 5-day coadministration of eltrombopag (75 mg once daily) significantly increased rosuvastatin *AUC* by 55% and C_{max} by 103%, respectively. The C_{max} of eltrombopag at steady-state following multidose (75 mg) was reported to 8.08 μM (Allred et al. 2011). Assuming that F_a and f_u were set to be 0.52 and 0.01 (Elsby et al. 2016), respectively, following oral multidose of eltrombopag (75 mg), $f_u \times I_{inlet,msx}$, $f_u \times C_{max}$, $I_{gut,max}$, and I_2 were estimated to be 0.24 μM, 0.08, 29.4 μM, and 678 μM, respectively. In the liver, the estimated values of both $f_u \times I_{inlet,max}$ and $f_u \times C_{max}$ were much less than IC_{50} for BCRP and OATP1B1 inhibition, whose R-values ($1 + f_u \times I_h/IC_{50}$) in the liver were below 1.1, but in the gut both *R*-values ($1 + I_{gut\ max}/K_I$ and $1 + I_2/K_I$) were higher than 11, indicating that increases in oral plasma exposure of rosuvastatin were mainly attributed to inhibition of intestinal BCRP and that inhibition of hepatic BCRP and OATP1B1 by oral eltrombopag was minor, which may also explain that oral administration of eltrombopag did not affect pharmacokinetics of ceftriaxone following intravenous ceftriaxone, a substrate of BCRP (Neves et al. 2018).

Antifolate drug MTX is a substrate of many transporters such as BCRP, OATs, MRP2, and MRP4. High doses of MTX are an accepted treatment for lymphoid malignancy, osteogenic sarcoma, and acute leukemia, while low doses of MTX often are used to the treatment of rheumatoid arthritis. MTX pharmacokinetics is highly variable. MTX can be absorbed via gastrointestinal tract, but its bioavailability is often decreased along with dose increases, due to limitation of absorption. Within cells, MTX is converted to active metabolite methotrexate polyglutamates. MTX is partly oxidized to 7-hydroxylmethotrexate, a less active metabolite. MTX is mainly eliminated via renal tubular excreted in intact drug. A series of clinical reports have demonstrated potentially toxic DDIs with many commonly used drugs including nonsteroidal anti-inflammatory drugs (NSAIDs), penicillins, and proton pump inhibitors (PPIs) or ciprofloxacin.

In clinic, NSAIDs may be comedicated with MTX. However, reports about DDIs of NSADIs with MTX are often contradictory. Joerger et al. (2006) reported that concurrent administration of NSAIDS resulted in a 16% decrease in CL_t of MTX and a 38% decrease in CL_t of 7-hydroxy-MTX following high oral dose (dose range 300 mg/m^2 to 12 g/m^2) to patients based on population pharmacokinetic analysis. Tracy et al. (1992) studied the pharmacokinetics of MTX in patients with rheumatoid arthritis concurrently treated with choline magnesium trisalicylate, ibuprofen, naproxen, or a non-NSAID analgesic (control treatment). They found that all three treatments significantly reduced the CL_t of MTX. Both trisalicylate and ibuprofen

also significantly reduced CL_r of MTX (Tracy et al. 1992). In 13 women and 2 men with rheumatoid arthritis receiving a stable dosage of MTX (maximum 20 mg/week) for at least 2 months, it was found that coadministration of aspirin (3.9 g) significantly lowered CL_t of MTX following intravenous dose (10 mg) from 70.6 ± 18.5 mL/min/m^2 to 59.2 ± 11.3 mL/min/m^2. Eleven of 14 patients had a decrease in CL_r of MTX when aspirin was coadministrated. The AUC^{24} (1634.0 ± 409.00 ng.h/mL) in patients receiving MTX with aspirin was greater than that (1272.43 ± 227.22 ng.h/mL) in patients without aspirin (Stewart et al. 1991). Several evidences have demonstrated that NSAIDs increase plasma exposure of MTX via renal excretion mediated by transporters. In rats, it was reported that NSAIDs, except salicylate, were potent inhibitors of rat Oat3 (K_i of 1.3–19 μM) (Nozaki et al. 2004). Takeda et al. (2002b) also reported that NSAIDs (salicylate, ibuprofen, ketoprofen, phenylbutazone, piroxicam, and indomethacin) dose-dependently inhibited uptake of MTX mediated by human OAT1, human OAT3, and human OAT4. The K_I values for the effects of salicylate, phenylbutazone, indomethacin, and probenecid on hOAT3-mediated MTX uptake were comparable with therapeutically relevant plasma concentrations of unbound drug (Takeda et al. 2002b). NSAIDs were also reported to significantly inhibit transport of MTX mediated by MRP2 and MRP4 (El-Sheikh et al. 2007). Salicylate and indomethacin were predicted to inhibit MRP4 at clinical plasma concentrations (Nozaki et al. 2007). NSAID-glucuronide (NSAID-Glu) and major metabolites of NSAIDs (diclofenac, ibuprofen, flurbiprofen, and naproxen) also exhibited concentration-dependent inhibitory effects on MTX uptake via OAT1 and OAT3, especially OAT3 (Iwaki et al. 2017). High levels of diclofenac-acyl glucuronide in plasma of human were also detected following oral diclofenac (50 mg). The ratios of diclofenac acyl glucuronide to diclofenac for C_{max} and AUC were 0.62 ± 0.21 and 0.84 ± 0.21, respectively. Diclofenac acyl glucuronide was also identified as a substrate of numerous OAT1, OAT2, OAT3, OAT4, and MRP2 (Zhang et al. 2016). These results indicate that DDIs of NSAIDs including their metabolites with MTX result from work in coordination with these transporter inhibitions.

Interestingly, DDIs of MTX with NSAIDs are dependent on dose of MTX. Kremer and Hamilton (1995) investigated effect of NSAIDs on pharmacokinetics of MTX at the 7.5 mg weekly dose of MTX in 30 patients. They found that coadministration of NSAIDs did not significantly alter pharmacokinetics of MTX, but in additional 16 patients, it was found that coadministration of NSAIDs significantly reduced the CL_r of MTX at usual weekly maintenance dose of MTX (mean 16.6 mg) (from 115.3 ± 34.4 mL/min to 91.7 ± 26.4 mL/min), accompanied by significant decrease in creatinine clearance (95.3 ± 26.3 mL/min to 77.5 ± 13.9 mL/min).

Recently, Hall et al. (2017) reviewed 32 articles about DDIs of low-lose MTX with NSAIDs, penicillins, or PPIs. They got a conclusion that although there are some pharmacokinetic data to describe increased MTX concentrations at low dose when NSAIDs are used concomitantly, the clinical relevance remains unclear. No clinically meaningful interaction of MTX at low dose with penicillins and PPIs was identified (Hall et al. 2017). Similarly, Zachariae (1992) thought that harmful

interactions of MTX with NSAIDs must be extremely rare when they were in low dose. The author suggested that it is a suitable selection to give a low dose of NSAID and to reduce the dosage of MTX when NSAIDs are given concomitantly (Zachariae 1992). In consistence, Colebatch et al. (2011) reviewed safety of MTX coadministrated NSAID aspirin, or paracetamol, on 17 publications out of 8681 identified studies in inflammatory arthritis. They got a conclusion that in the management of rheumatoid arthritis, the concurrent use of NSAIDs with MTX appears to be safe, and appropriate monitoring is necessary, but the use of anti-inflammatory doses of aspirin should be avoided. In spite of this, it should be noted that comedication of NSAIDs whit high-dose MTX may enhance MTX toxicity due decrease in renal MTX excretion rate (Fischetti 1990).

PPIs are often comedicated with MTX. Obvious DDIs mainly occur at high dose of MTX. Bezabeh et al. (2012) reviewed cases about DDIs of MTX with PPIs reporting both in FDA Adverse Event Reporting System and the published literatures. They found that concomitant use of MTX (primarily at high doses) with PPIs (such as omeprazole, esomeprazole, and pantoprazole) decreased CL_r of MTX, leading to elevated plasma levels of MTX and/or hydroxymethotrexate, possibly increasing MTX toxicities. Based on the reviewed data, the FDA has updated the MTX label that the possible DDIs of high-dose MTX with PPIs occur. Santucci et al. (2010a) also analyzed the impact of PPI coadministration on MTX elimination in cancer patients receiving treatment with high dose of MTX (>1 g/m^2 intravenously) in 79 patients for 197 cycles. It was found that delayed MTX elimination (i.e., plasma concentration >15 μM at 24 h, >1.5 μM at 48 h and/or > 0.15 μM at 72 h) occurred in 16% (32/197) of the cycles. The co-prescription of a PPI (pantoprazole, lansoprazole, omeprazole, or esomeprazole) was found in 53% (17/32) of the courses with delayed elimination and in 15% (24/165) of the cycles without delayed elimination. In a retrospective study, it was found that patients who received a PPI had significantly higher MTX levels at 48 h (0.38 μM versus 0.15 μM) and 72 h (0.13 μM versus 0.05 μM) compared with patients without PPI. In consistence, patients receiving PPIs had significantly longer infusion times (Narumi et al. 2017). In 74 patients receiving high-dose MTX coadministrated PPIs, Suzuki et al. (2009) found that concentrations of plasma MTX (11.5 μM, 0.87 μM, and 0.23 μM, respectively) at 24, 48, and 72 h in patients with PPIs were significantly higher than those (1.1 μM, 0.11 μM, and 0.05 μM, respectively) without PPIs. Correspondingly, abnormally high serum creatinine and serum AST and ALT concentrations were significantly more frequent in the delayed elimination group than in the normal elimination group. Multiple logistic regression analysis revealed that concurrent administration of PPIs increases the risk of delayed MTX elimination by 2.65 times, accompanied by higher plasma MTX concentration in high-dose MTX therapy. These results indicate that coadministration of PPIs may be identified as a risk factor for delayed elimination of MTX in high-dose MTX therapy (Santucci et al. 2010a; Suzuki et al. 2009). Thus, physicians should note this potential DDIs in patients receiving concomitant high-dose MTX and PPIs to avoid seriously adverse events. For example, Santucci et al. (2010b) reported that among six patients showing delayed elimination of MTX associated with impaired renal function, five

cases possibly came from DDIs including the coadministration of piperacillin/tazobactam ($n = 1$) and proton pump inhibitors (omeprazole, $n = 3$; esomeprazole, $n = 2$). Similarly, seriously adverse events were reported by McBride et al. (2012) in a patient with osteosarcoma receiving omeprazole with high-dose MTX for stress ulcer prophylaxis. Beorlegui et al. (2000) also reported a case of delayed elimination of high-dose MTX associated with concomitant omeprazole administration. Naranjo probability scale demonstrated a probable DDI of MTX and omeprazole.

In general, multiple renal transporters are involved in renal tubular secretion of MTX including BCRP, MRPs, and OATs. Omeprazole, lansoprazole, rabeprazole, and pantoprazole showed potential inhibition on MTX (Suzuki et al. 2009). Pantoprazole was reported to inhibit MTX transport via inhibition of BCRP but not MRP2 (Breedveld et al. 2004). Moreover, omeprazole, lansoprazole, rabeprazole, and pantoprazole were reported to inhibit BCRP-mediated transport of MTX (Suzuki et al. 2009). The IC_{50} values of BCRP inhibition were 17.6 μM for omeprazole, 14.4 μM for lansoprazole, 8.5 μM for rabeprazole, and 5.5 μM for pantoprazole, respectively, which were much higher than unbound plasma levels ($f_u C_{\max}$) of PPIs (0.090, 0.081, 0.045, and 0.109 μM, respectively) in the therapeutic range. The R-values ($1 + f_u C_{\max}/IC_{50}$) were less than 1.1, indicating that DDIs of MTX with PPIs are unlikely to be the result of the inhibitory effect of PPIs on renal apical BCRP-mediated MTX transport. Renal OAT1 and OAT3 have been shown to transport MTX. Esomeprazole, lansoprazole, omeprazole, and rabeprazole were also reported to significantly inhibit hOAT3-mediated uptake of MTX (Narumi et al. 2017). The observed IC_{50} values of esomeprazole, lansoprazole, omeprazole, and rabeprazole for the inhibition of MTX uptake were 1.2, 0.40, 5.5, and 4.8 μM, respectively. The $C_{\max}$ of esomeprazole at steady-state following 40 mg (Shin et al. 2014) and plasma binding (Andersson et al. 2001) were reported to 4.15 μM and 97%, respectively. Thus, except lansoprazole, IC_{50} of other three PPIs are also much higher than their $f_u C_{\max}$ values in the therapeutic range (R-values below 1.1). DDI between MTX and PPIs cannot be explained solely by the inhibitory effects of PPIs on renal OAT3 or BCRP, which should be attributed to synergistic effect of these transporter inhibitions, which need further investigation. $f_u C_{\max}$ value of lansoprazole was reported to be 0.081 μM (Suzuki et al. 2009) at 30 mg; thus, R-value ($1 + f_u C_{\max}/IC_{50}$) was calculated to 1.2, larger than 1.1, which seemed to partly explain DDI of MTX with lansoprazole.

However, no obvious interactions of MTX with PPIs are demonstrated in several reports. For example, Ranchon et al. (2018) investigated the incidence of delayed MTX elimination in patients treated with high-dose MTX (≥ 1 g/m^2) for hematological malignancy to identify the impact of interacting drugs, especially PPIs and ranitidine. A total of 412 cycles of MTX were administered to 179 patients. PPIs were coadministered with MTX in 127 cycles and ranitidine in 192 cycles. Ninety-three cycles included no antacid drugs. No association of PPIs (pantoprazole and esomeprazole) or ranitidine and delayed MTX elimination was found. Similarly, Reeves et al. (2014) assessed potential DDI between MTX and PPIs in patients receiving high-dose MTX using 56 adults receiving 201 cycles of MTX. They found that despite a significant difference between those receiving a PPI and not

receiving a PPI in median MTX levels at 24 h (8.0 μM versus 3.9 μM) and 72 h (0.08 μM versus 0.05 μM) after MTX administration, there was no difference between those receiving a PPI and not receiving a PPI in the proportion of patients experiencing delayed elimination at 24 h (19.2% versus 20.2%, respectively) and 72 h (36.2% versus 33.7%). They did not find a significant interaction between PPIs and MTX. These contradictory results may come from use of different PPIs, MTX dosage, or renal function of patients, which need further investigation. Facts that different PPIs show greatly different inhibition on OAT- or BCRP-mediated MTX transport seem also to explain these contradictory reports.

Moreover, concomitant administration of penicillins (Yamamoto et al. 1997) or ciprofloxacin (Dalle et al. 2002; Jarfaut et al. 2013; Kamangar et al. 2013) delayed MTX elimination, which is partly attributed to inhibition of renal OAT3-mediated MTX excretion (Vanwert et al. 2018).

5.7 Fruit-Drug Interaction and Their Clinical Significance

In addition to DDIs, there are numerous patients who encounter increased risks of adverse events associated with drug-food interactions. Grapefruit juice is one of the most intensively studied dietary substances, which significantly increases plasma drug exposure in humans by up to 16-fold (Hanley et al. 2011). Grapefruit juice-drug interaction was first identified by Bailey et al. (1989). They investigated the interaction between ethanol and felodipine in ten volunteers and found that felodipine plasma concentrations greatly increased in volunteers treated with grapefruit juice used as a vehicle. Since then, a series of studies on grapefruit juice-drug interactions have been demonstrated in human. Pharmacokinetic studies have demonstrated that grapefruit juice can increase the bioavailability of some therapeutic drugs including calcium channel blockers, benzodiazepines, and statins, leading to the potential to interact with grapefruit and cause serious adverse effects including torsade de pointes, rhabdomyolysis, myelotoxicity, respiratory depression, gastrointestinal bleeding, or nephrotoxicity (Bailey et al. 2013). These drugs are often substrates of CYP3A4 or P-GP and undergo extensively presystemic extraction. Grapefruit juice increases oral plasma exposure of drug via affecting intestinal CYP3A4 and intestinal P-GP.

Widely investigated drugs are dihydropyridine calcium channel blockers including felodipine, nifedipine, and nisodipine. In six men with borderline hypertension, it was found that intake of grapefruit juice significantly increased felodipine bioavailability to 284% of that with water. The dehydrofelodipine/felodipine *AUC* ratio was decreased. In six healthy men, intake of grapefruit juice also increased the nifedipine bioavailability by 34% of that with water (Bailey et al. 1991). Single intake and 14-day intake of grapefruit juice showed similar increase effects on oral plasma exposure of felodipine (Lundahl et al. 1998). It was consistent with increased plasma exposure of felodipine that intake of grapefruit juice resulted in more

pronounced hemodynamic effects of the drug than water. The vascularly related adverse events were more frequent during treatment with grapefruit juice (Bailey et al. 1991; Lundahl et al. 1997, 1998). Importantly, intake of grapefruit juice did not significantly alter the intravenous pharmacokinetics of felodipine, indicating that grapefruit juice increased the plasma concentrations of felodipine mainly via inhibition of gut wall metabolism (Lundahl et al. 1997). Effects of grapefruit juice on intestinal and hepatic CYP3A4 activity in ten healthy men were also investigated (Lown et al. 1997). It was found that the 6-day intake of grapefruit juice did not alter liver CYP3A4 activity ([^{14}C] N-methyl-erythromycin breath test), colon levels of CYP3A5, or small bowel concentrations of P-GP, villin CYP1A1, and CYP2D6. But the concentration of CYP3A4 in small bowel epithelia was decreased to 62% of those before grapefruit juice intake, demonstrating selective downregulation of CYP3A4 in the small intestine by grapefruit juice (Lown et al. 1997).

Statins, especially simvastatin, have been also widely investigated. In a randomized crossover study with two phases, ten healthy volunteers ingested grapefruit juice 200 mL (200 mL normal-strength grapefruit juice once a day) for 3 days (Lilja et al. 2004). On day 3, a single 40 mg dose of simvastatin was administered with grapefruit juice 200 mL. The results showed that compared with water, intake of grapefruit juice significantly increased *AUC* of simvastatin 3.6-fold and that of simvastatin acid 3.3-fold, respectively. The C_{max} of simvastatin and simvastatin acid during grapefruit juice were increased 3.9-fold and 4.3-fold, respectively. Three-day intake of grapefruit juice also was reported to significantly increase *AUC* of atorvastatin 2.5-fold of that with water, whereas its C_{max} was not significantly changed. The *AUC* of atorvastatin lactone was increased 3.3-fold and the C_{max} 2.6-fold. In line with inhibition of CYP3A4, C_{max} and *AUC* of 2-hydroxyatorvastatin acid and 2-hydroxyatorvastatin lactone were also significantly decreased (Lilja et al. 1999). However, intake of grapefruit juice has minimal effects on pharmacokinetics of pravastatin (Lilja et al. 1999) and pitavastatin (Ando et al. 2005), which are minimally metabolized by CYP3A4.

The increases in plasma exposure of statins are dependent on strength of grapefruit juice. Two reports demonstrated that 3-day double-strength grapefruit juices (i.e., 200 mL of grapefruit juice three times a day for 2 days. On day 3, simvastatin was administrated with 200 mL grapefruit juice, and an additional 200 mL was ingested 0.5 and 1.5 h following simvastatin dose) significantly increased C_{max} and *AUC* of simvastatin about 9~12-fold and 13.5~16-fold of those with water (Lilja et al. 1998a, 2009). The mean C_{max} and *AUC* of simvastatin acid were also both increased about sevenfold (Lilja et al. 1998a). Similarly, the 3-day intake of grapefruit juice (an 8 ounce glass of regular-strength grapefruit juice once a day) only increased the *AUC* and C_{max} of lovastatin by 30~40% of those with water (Rogers et al. 1999), but 3-day double-strength grapefruit juice (i.e., 200 mL grapefruit juice three times a day for 2 days. On day 3, lovastatin was administrated with 200 mL grapefruit juice, and an additional 200 mL was ingested 0.5 and 1.5 h following lovastatin dose) greatly increased the serum concentrations of both lovastatin and lovastatin acid. The C_{max} and *AUC* of lovastatin were increased about 12-fold and fivefold of those with water,

respectively. The mean C_{max} and AUC of lovastatin acid were increased about fourfold and fivefold, respectively (Kantola et al. 1998). A clinical report demonstrated that intake of grapefruit juice with atorvastatin or simvastatin resulted in severe rhabdomyolysis (Bailey et al. 2013; Mazokopakis 2008).

Grapefruit juice also increases oral plasma exposure of other drugs. For instance, compared with water, intake of grapefruit juice significantly increased halofantrine AUC and C_{max} by about 2.8-fold and 3.2-fold, respectively. There was a concomitant 2.4-fold decrease in its metabolite N-debutyl-halofantrine AUC. Correspondingly, maximum QTc interval prolongation induced by halofantrine increased from 17 ± 6 ms during water phase to 31 ± 12 ms during grapefruit juice phase, indicating that grapefruit juice should be contraindicated during administration of halofantrine (Charbit et al. 2002). Intake of grapefruit juice also increased the C_{max} of buspirone 4.3-fold and AUC 9.2-fold of those with water (Lilja et al. 1998b).

Grapefruit juice is rich in a number of phytochemicals, including flavonoids and furanocoumarins. The most abundant flavonoid in the juice is naringin. These phytochemicals are considered to be inhibitors of intestinal CYP3A, intestinal OATP1A2, or intestinal OATP2B1. Due to their abundance and in vitro inhibition of CYP3A, these flavonoids were originally presumed to be the grapefruit juice constituents responsible for mediating DDIs. However, in vivo data showed that although intake of grapefruit juice significantly increased oral plasma exposures of felodipine, nisoldipine, and nifedipine in human, oral administration of naringin, flavonoid, or quercetin little altered pharmacokinetic profiles of the three drugs (Bailey et al. 1993, 1998; Rashid et al. 1993), indicating that these flavonoids are not thought to play a major role in grapefruit juice-drug interactions involving CYP3A substrates. Furanocoumarins are a structurally distinct class of compounds found in grapefruit juice including bergaptol, bergapten, spiroesters, bergamottin, and 6′,7′-dihydroxybergamottin, among which bergamottin and 6′,7-′-dihydroxybergamottin have been the most extensively studied. Bergamottin and 6′,7′-dihydroxybergamottin belong to mechanism-based inhibitors of CYP3A4. However, a report showed that bergamottin and 6′,7′-dihydroxybergamottin at 10 μM inhibited midazolam 1′-hydroxylation activity in Caco-2 cells by 34% and 93% (Malhotra et al. 2001), indicating that 6′,7′-dihydroxybergamottin shows a stronger inhibition on CYP3A4. Moreover, concentration of 6′,7-′-dihydroxybergamottin was also higher than that of bergamottin (Malhotra et al. 2001). These results suggest that 6′,7′-dihydroxybergamottin in grapefruit juice is more important for in vivo inhibition of CYP3A than bergamottin. In vivo data have demonstrated that furanocoumarins are responsible for the effects of grapefruit juice on some drugs. It was reported that compared with common orange juice, intake of grapefruit juice, but not with furanocoumarin-free grapefruit juice, significantly increased cyclosporine AUC and C_{max} (Paine et al. 2008). Concentrations of bergamottin (5 μM) and 6′,7′-dihydroxybergamottin (36 μM) in Seville orange juice were reported to be comparable to those (16 μM and 23 μM) in dilute grapefruit juice (Malhotra et al. 2001). In consistence, compared with common orange juice,

intake of Seville orange juice and diluted grapefruit juice showed similar effects on oral plasma exposure of felodipine (Malhotra et al. 2001). However, although pure bergamottin enhanced the oral bioavailability of felodipine, increases in bioavailability of felodipine by administration of pure bergamottin (12 mg) were substantially less than that by grapefruit juice (containing1.7 mg bergamottin) (Goosen et al. 2004). Similarly, Bailey et al. (1998) compared the effects of grapefruit juice, supernatant fraction (containing 148 mg naringin and 1.85 mg 6′,7-′-dihydroxybergamottin), and particulate fractions (naringin 7 mg and 6′,7-′-dihydroxybergamottin 0.60 mg) on oral plasma exposure of felodipine. They found that increases in *AUC* of felodipine were grapefruit juice (2.45-fold) > particulate fraction (2.21-fold) > supernatant fraction (1.52-fold). Similar orders were found in the increased C_{max} of felodipine, indicating that the increases in plasma exposure of felodipine were not consistent with amount of naringin and 6′,7-′-dihydroxybergamottin. Different from findings in felodipine, intake of Seville orange juice had no influence on cyclosporine disposition, although *AUC* and C_{max} of cyclosporine during grapefruit juice were increased by 55% and 35%, respectively. In vitro data demonstrated that 6′,7′-dihydroxybergamottin still inhibited CYP3A4 activities and did not inhibit P-GP at concentrations up to 50 μM, indicating that 6′,7′-dihydroxybergamottin is not responsible for the effects of grapefruit juice on cyclosporine (Edwards et al. 1999). All these results indicate bergamottin and 6′,7′-dihydroxybergamottin are not sole major active ingredients leading to the grapefruit juice-drug interaction. It appears probable that the interaction also involves other furanocoumarins present in whole grapefruit juice, possibly acting in combination by additive or synergistic mechanisms, which need further investigation.

On the other hand, grapefruit juice can remarkably decrease intestinal absorption of some therapeutic drugs such as fexofenadine, celiprolol, talinolol, etoposide, montelukast, and aliskiren. These drugs are often substrates of OATP1A2 or OATP1B2. Banfield et al. (2002) first reported that intake of grapefruit juice significantly reduced C_{max} and *AUC* of fexofenadine by 30% of those with water. It was also found that decrease in bioavailability of fexofenadine was dependent on volume intake of grapefruit juice (Dresser et al. 2005). Compared with water, intake of 300-mL volume grapefruit juice decreased *AUC* and C_{max} of fexofenadine by 42% and 47%, respectively, and 1200 mL grapefruit juice reduced *AUC* and C_{max} by 64% and 67%, respectively. Moreover, ingestion of grapefruit juice stereoselectively altered oral pharmacokinetics of fexofenadine and induced decreases in *AUC* for (*R*)- and (*S*)-fexofenadine by 39% and 52%, respectively. Subsequently, grapefruit juice increased the mean R/S ratio of the *AUC* from 1.58 to 1.96. Although grapefruit juice greatly reduced the amounts of (*R*)- and (*S*)-fexofenadine excreted into the urine by 52% and 61%, respectively, the mean *R/S* ratios of excreted amount in urine during 24 h and the CL_r of both enantiomers were unchanged (Akamine et al. 2015). Intake of grapefruit juice significantly decreased bioavailabilities of other OATP1A2 or OATP2B1 substrates such as talinolol (Schwarz et al. 2005), etoposide (Reif et al.

2002), montelukast (Cingi et al. 2013), celiprolol (Lilja et al. 2003), and aliskiren (Rebello et al. 2012; Tapaninen et al. 2010). For example, intake of grapefruit juice was reported to significantly reduce the mean *AUC*, C_{max} and cumulative urinary excretion of celiprolol to about 13%, 5% and 15%, respectively, of these with water (Lilja et al. 2003). Similarly, intake of grapefruit juice also decreased the plasma concentration of aliskiren with mean decreases in *AUC* and C_{max} of 38% and 61%, respectively, of those with water (Rebello et al. 2012).

Grapefruit juice is rich in flavonoids, which of the most abundant flavonoids is naringin. In vitro data showed that naringin concentration dependently inhibited uptake of aliskiren and fexofenadine in OATP1A2-expressing cells with mean IC_{50} values of 75.5 and 24.2 μM (Dresser et al. 2002). The inhibition of intestinal OATP1A2 by naringin is considered to be a reason decreasing bioavailabilities of OATP1A2 substrates. Bailey et al. (2007) investigated the roles of naringin in decreased oral plasma exposure of fexofenadine by grapefruit juice using two randomized, crossover, pharmacokinetic studies. In one study, 120 mg of fexofenadine was ingested with 300 ml grapefruit juice, an aqueous solution of naringin at the same juice concentration (1200 μM) or water. In the other study, fexofenadine was administered with suspension of the particulate fraction of juice containing known clinical inhibitors of enteric CYP3A4, but relatively low naringin concentration (34 μM), or with water. They found that fexofenadine *AUC* with grapefruit juice and naringin solution were 55% and 75% of that with water, respectively. But particulate fraction containing inhibitors of enteric CYP3A4 or low levels of naringin did not affect *AUC* of fexofenadine. These results demonstrated that naringin most probably directly inhibited enteric OATP1A2 to decrease oral fexofenadine bioavailability. In consistence, intake of grapefruit, orange, and apple juices decreased the fexofenadine *AUC*, C_{max}, and the urinary excretion values to 30% to 40% of those with water (Dresser et al. 2002). Won et al. (2013) investigated the effects of a modified grapefruit juice devoid of furanocoumarins (~99%) and polymethoxyflavones (~90%) on fexofenadine disposition in human. They found that administration of grapefruit juice and modified grapefruit both juices decreased fexofenadine geometric mean *AUC* and C_{max} by about 25%, indicating that furanocoumarins and polymethoxyflavones are not major mediators of the grapefruit juice-fexofenadine interaction.

Other fruit juices may lead to fruit-drug interaction. Intake of orange juice reduced the mean C_{max}, mean *AUC*, and urinary excretion of celiprolol by 89%, 83%, and 77% (Lilja et al. 2004), respectively. Ingestion of apple juice significantly decreased *AUC* for (*R*)- and (*S*)-fexofenadine by 49% and 59%, respectively. In oocytes expressed OATP2B1, it was found apple juice significantly decreased the uptake of both enantiomers (Akamine et al. 2014). Concomitant intake of kola nut significantly decreased plasma concentrations of halofantrine and the active metabolite desbutylhalofantrine following a single dose of 500 mg halofantrine hydrochloride, inducing decrease in halofantrine C_{max} by 45% and *AUC* by 32% and decrease in desbutylhalofantrine C_{max} by 50% and *AUC* by 45% (Kolade et al. 2008). Table 5.2 lists several drugs that possibly interact with grapefruit juice and its possible adverse event related to drugs.

Table 5.2 Selected drugs that possibly interact with grapefruit juice and altered oral bioavailability and its possible adverse events

Indicated drugs	Altered bioavailability	Dose-related adverse event
Cardiovascular agents		
Amiodarone	↑	Torsade de pointes
Felodipine	↑	Hypotension
Nifedipine	↑	Hypotension
Manidipine	↑	Hypotension
Nitrendipine	↑	Hypotension
Pranidipine	↑	Hypotension
Nisodipine	↑	Hypotension
Verapamil2	↑	Hypotension, bradyarrhythmias
Quinidine	↑	Hypotension, torsade de pointes
Ticagrelor	↑	Gastrointestinal or kidney bleeding
Aliskiren	↓	Loss or decrease of efficacy
Celiprolol	↓	Loss or decrease of efficacy
Talinolol	↓	Loss or decrease of efficacy
CNS agents		
Buspirone	↑	Dizziness, sedation
Pimozide	↑	Torsade de pointes
Midazolam	↑	Dizziness, sedation
Triazolam	↑	Dizziness, sedation
Alprazolam	↑	Dizziness, sedation
Diazepam	↑	Dizziness, sedation
Immunomodulators		
Cyclosporine	↑	Nephrotoxicity
Everolimus	↑	Myelotoxicity, nephrotoxicity
Sirolimus	↑	Myelotoxicity, nephrotoxicity
Tacrolimus	↑	Nephrotoxicity
Antilipemic agents		
Atorvastatin	↑	Rhabdomyolysis
Lovastatin	↑	Rhabdomyolysis
Simvastatin	↑	Rhabdomyolysis
Anti-infective agents		
Erythromycin	↑	Torsade de pointes
Halofantrine	↑	Torsade de pointes
Quinine	↑	Torsade de pointes
Others		
Montelukast	↓	Loss or decrease of efficacy
Etoposide	↓	Loss or decrease of efficacy
Colchicine	↑	Myelotoxicity
Fexofenadine	↓	Loss or decrease of efficacy
Cisapride	↑	Torsade de pointes

Taken together, some fruit juices including grapefruit, apple, and orange alter oral disposition of drugs by affecting activity and expression of intestinal OATP1A2, CYP3A4, or P-GP. However, further studies are needed to optimize our knowledge on specific ingredients of juices and their roles in regulation of intestinal transporter expression as well as the clinical significance of the fruit-drug interactions.

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Chapter 6
Roles of Hepatic Drug Transporters in Drug Disposition and Liver Toxicity

Guoyu Pan

Abstract Hepatic drug transporters are mainly distributed in parenchymal liver cells (hepatocytes), contributing to drug's liver disposition and elimination. According to their functions, hepatic transporters can be roughly divided into influx and efflux transporters, translocating specific molecules from blood into hepatic cytosol and mediating the excretion of drugs and metabolites from hepatic cytosol to blood or bile, respectively. The function of hepatic transport systems can be affected by interspecies differences and inter-individual variability (polymorphism). In addition, some drugs and disease can redistribute transporters from the cell surface to the intracellular compartments, leading to the changes in the expression and function of transporters. Hepatic drug transporters have been associated with the hepatic toxicity of drugs. Gene polymorphism of transporters and altered transporter expressions and functions due to diseases are found to be susceptible factors for drug-induced liver injury (DILI). In this chapter, the localization of hepatic drug transporters, their regulatory factors, physiological roles, and their roles in drug's liver disposition and DILI are reviewed.

Keywords Hepatic drug transporters · Drug disposition · Hepatotoxicity · DILI

6.1 Introduction

It is reported that liver accounts for more than 70% of drug metabolism and elimination in clinic (Patel et al. 2016). As the major site of drug metabolism, the parenchymal liver cells (hepatocytes) occupy almost 80% of the total liver volume and 60% of the total number of liver cells (Kmiec 2001). For non-parenchymal liver

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cells like endothelial cells, cholangiocytes, and macrophages, they only contribute to approximately 6.5% of the liver volume.

Most of drug transporters are distributed in hepatocytes and contributed to drug's liver disposition and elimination. Liver membrane transporters have important effects on drug exposure and efficacy as well as metabolizing enzymes, and they also play unique roles in drug-induced liver injury (DILI).

6.2 Major Hepatic Drug Transporters and Their Distribution

6.2.1 Classification and Features of Hepatic Transporters

Hepatic transporters can be roughly divided into two parts according to their functions. Influx transporters translocate specific molecules from blood into hepatic cytosol, mainly expressed in the basolateral membrane of hepatocytes (Akira 2006, Shitara and Sugiyama 2006, Evers and Chu 2008). Efflux transporters mediate the excretion of drugs and metabolites from hepatic cytosol to blood and bile, distributed in the basolateral membrane and canalicular membrane of hepatocytes, respectively. However, as discussed in the following sections, many transporters (especially uptake transporters) have bidirectional transport ability under specific cellular physiological conditions. If classified by location, hepatic transporters consist of basolateral transporters and canalicular transporters (Priyamvada and Kim 2004). Figure 6.1. provides an overview of the locations of major hepatic transporters.

6.2.1.1 Hepatic Influx Transporters

Several transport proteins involved in influx transporters have been identified, including the organic anion transporters (OATs), organic cation transporters (OCTs), organic

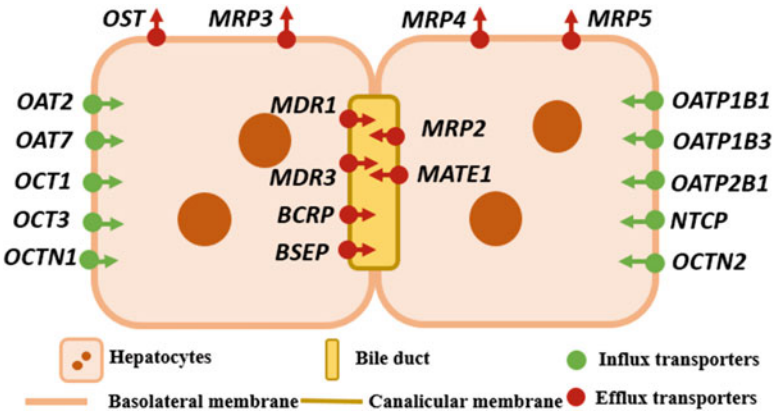

Fig. 6.1 Schematic figure of human hepatic basolateral and canalicular transport proteins

anion transporting polypeptides (OATPs), and Na + −taurocholate co-transporting polypeptide (NTCP) (Xu et al. 2014). The hepatic influx transport proteins belong to the gene superfamily of solute carriers (SLC), which can be distinguished into uniporters and secondary or tertiary active transporters (Dobson PD 2008). Uniporters provide facilitated diffusion down the electrochemical gradient, while secondary or tertiary active transporters can translocate substrates against their concentration gradient with a driving force from co- or counter-transport of inorganic and small organic ions (Klaassen and Aleksunes 2010a). In addition, some influx transporters are also involved in bidirectional transport, such as OAT2 and OATPs.

6.2.1.1.1 OATPs

OATPs are a family of proteins that plays crucial roles in the hepatic clearance of a large range of substrates, including organic anions, some type II cations, and neutral steroids (Priyamvada and Kim 2004). Unlike other influx transporters, all the members of OATPs can transport both endogenous and xenobiotic compounds bidirectionally, such as bile acids, prostaglandins, cyclic nucleotides, and their conjugates (Thakkar et al. 2015). Many clinical drugs have been determined to be substrates or inhibitors of OATPs, which may lead to side effects (Niemi et al. 2011) or drug-drug interactions (Huang et al. 2014).

In human liver, OATP1A2, OATP1B1, OATP1B3, and OATP2B1 are the primary members of OATPs. The International Transporter Consortium (ITS) recommends to investigate the four OATPs due to their significant functions in drug disposition and metabolism (Kovacsics et al. 2017).

OATP1B1, OATP1B3, and OATP2B1 are expressed on the basolateral membrane of hepatocytes, delivering drugs from blood into the cells (Shitara et al. 2013), while OATP1A2 is localized on the membrane of cholangiocytes, contributing to the reabsorption of bile acid, unconjugated bilirubin, and xenobiotic (Hagenbuch and Stieger 2013). OATP1B1 and OATP1B3 are two liver-specific OATPs. If the two OATPs are found in organs besides liver, it suggests that these organs may be turning cancerous. So they can be used as biomarkers of cancer (Huang et al. 2014). On the other hand, it was reported that there was a reduction in the expression of OATP1B1 and OATP1B3 in hepatoma cancer cells compared to normal liver cells (Monks Nr et al. 2007). Many anticancer drugs have been characterized as the substrates of OATPs (Huang et al. 2014), which provide a new anticancer drug development strategy by increasing the bioavailability of drugs by activating OATP1B1 and OATP1B3.

These four OATPs share a lot of substrates/inhibitors (Giacomini et al. 2012), such as cholephilic organic anion bromosulfophthalein (BSP) and dehydroepiandrosterone sulfate (DHEAS) and estrone-3-sulfate (Kullak-Ublick et al. 2001), which make it difficult to justify their relative contributions to their substrate liver uptake. OATP1B1 is the most abundant OATP transporter in mRNA and protein expression levels (Kunze et al. 2014). It is responsible for the majority of Na +-

independent bile salt uptake (Kullak-Ublick et al. 2001) and transport both conjugated and unconjugated bilirubin (Cui et al. 2001) and all statins (Niemi et al. 2011). OATP1B3 also shows transport activity for conjugated and unconjugated bilirubin, but its affinity for unconjugated bilirubin is weaker than OATP1B1 (Niemi et al. 2011). If OATP1B1 is inhibited, OATP1B3 cannot completely compensate the lack of bilirubin uptake function in the liver, which will lead to unconjugated hyperbilirubinemia (Huang et al. 2014). In a lot of cases, OATP1B3 affinity to its substrate is weaker than OATP1B1 (Sheng et al. 2015). In addition, OATP1B3 is the major anionic peptide uptake system in human liver and exclusively transports digoxin (Priyamvada and Kim 2004).

6.2.1.1.2 OATs

The substrates of OATs are mainly organic anion compounds of small hydrophilic molecules, including many frequently used medicines in clinic, such as diuretics, antibiotics, nonsteroidal anti-inflammatory drugs (Dh 2005). In this family, OAT2 and OAT7 are expressed in the human liver, and the former dominated over the latter at the mRNA level (Sun et al. 2001, Shin et al. 2007).

OAT2 is expressed at the highest level in human liver, with lower expressions in kidney, lung, and many other tissues, so it is considered to be important in hepatic organic anion transport (Bleasby et al. 2006). Besides a wide variety of xenobiotics that have been mentioned above, OAT2 is able to transport numerous endogenous compounds like prostaglandin E2 and prostaglandin F2 DHEA sulfate (Enomoto et al. 2002, Kobayashi et al. 2005b, 2014, Cropp et al. 2008, Jia et al. 2015). Specially, OAT2 can transport cGMP in both directions. Cropp et al. found that OAT2 could uptake cGMP efficiently under normal condition while excreting it when there was an outward concentration gradient. It is possible that OAT2 may play a part in cGMP-signaling pathways (Cropp Cd et al. 2008). Recently, OAT2 has been found to mediate the uptake of antineoplastic drugs like irinotecan (Marada et al. 2015) and anti-hepatitis B virus drug named entecavir (Furihata et al. 2017). It was found that the decrease of OAT2 expression in noncancerous liver tissue can be used to predict the risk for HCC (hepatocellular carcinoma) development in chronic HCV (hepatitis C virus) patients (Yasui et al. 2014).

OAT7 is a newly found hepatic OAT. It is liver-specific and exclusively expressed in the basolateral membrane of hepatocytes (Shin et al. 2007). Moreover, there are no counterparts in mouse or rat (Jacobsson JA 2007). Compared to OAT2, OAT7's substrates are limited to sulfate conjugates, such as estrone sulfate (ES) and dehydroepiandrosterone sulfate (Shin et al. 2007). Notably, OAT7 is unique for its ability to transport ES in exchange for butyrate, which is not inhibited by typical OAT inhibitors like probenecid (Shin et al. 2007). Recently, Emami et al. reported that OAT7 is a novel pravastatin uptake transporter (Emami Riedmaier et al. 2016).

6.2.1.1.3 OCTs

OCT1 and OCT3 have been determined to locate in the basolateral membrane of human liver. The substrates of OCT1 are mainly monovalent and some divalent organic cations, as well as some weak bases and non-charged compounds (Koepsell 2013), such as tetraethylammonium (TEA) (L.Zhang et al. 1997) and 1-methyl-4-phenylpyridinium (MPP) (tranV. Gorboulev et al. 1997), two classic cationic compounds. The substrates of OCT3 are mainly limited to endogenous compound and neurotransmitter. Because of the overlap of substrates between OCT1 and OCT3, it is speculated that the function of OCT3 may be affected when OCT1 is inhibited or blocked (Nies 2010).

Previously reports indicated that express levels of OCT1/3 may be affected by cholestasis and hepatoma (NiesAT 2009, Heise M 2012). In the latter condition, the express levels of OCT1 and OCT3 have a distinct decrease, which could be used as biomarker to monitor the development of liver cancer (Schaeffeler et al. 2011).

Interestingly, sorafenib, which is employed to treat advanced hepatocellular carcinoma (Hong and Nam 2014), is a substrate of OCT1 (Lozano et al. 2013). Therefore, the effect of sorafenib is limited because of the impaired activity of this transporter in liver cancer patients. For patients treated with sorafenib, the OCT1 expression in liver tumors is a significant positive prognostic factor (Grimm et al. 2016).

In addition, OCTNs are a novel subfamily of OCTs (Wu et al. 1998), which are unique to recognize and transport zwitterionic carnitine (Tamai 2013), contributing to maintain homeostasis. In human, two isoforms have been identified in the liver, OCTN1 (Tamai et al. 1997) and OCTN2 (Tamai et al. 1998). The former is reported to be expressed only in fetal liver.

6.2.1.1.4 NTCP

NTCP is exclusively expressed in the basolateral membrane of hepatocytes, responsible for sodium-dependent bile salt uptake. Eighty percent of conjugated bile salts and 50% of unconjugated bile salts are absorbed by NTCP (Hagenbuch 2004). Besides, NTCP transports some non-bile salt substrates as well, such as DHEAS (Kullak-Ublick et al. 2000), thyroxine (T4) (Friesema et al. 1999), BSP, and estrone 3-sulfate (Meier et al. 1997).

Notably, NTCP has recently been identified as a specific receptor of hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis D virus (HDV), which mediates viral entry into the cell by specifically interacting with the pre-S1 domain of the L proteins, facilitating the infection of virus (Verrier ER 2016). Many efforts have been made to inhibit NTCP in purpose to prevent hepatitis B, C, and D virus infection. For example, Nkongolo et al. found that cyclosporin A could inhibit HBV/HDV infections during virus inoculation by blocking the binding site of virus on NTCP (Nkongolo et al. 2014). The inhibition of NTCP may lead to adverse drug interactions, especially if both NTCP and BSEP are blocked. For example,

troglitazone, glibenclamide, and rifampin may induce cholestasis by inhibiting NTCP and BSEP (Just and Upeslacis 1989). Wolf et al. found that several known hepatotoxic drugs such as ampicillin and carbenicillin inhibited NTCP without affecting BSEP (Wolf et al. 2010). Mita et al. demonstrated that cyclosporin A inhibited bile acid transport mainly by the inhibition of NTCP rather than BSEP (Mita et al. 2006). However, Dong et al. investigated 94 drugs, and the results indicated that there was no relationship between NTCP inhibition and DILI risk (Dong et al. 2015). The conclusion highlighted the feasibility to treat HBV via NTCP inhibition.

6.2.1.2 Hepatic Efflux Transporters

Hepatic efflux transporters consist of multidrug resistance proteins (MDRs), multidrug resistance-associated proteins (MRPs), breast cancer resistance protein (BCRP), bile salt export pump (BSEP), multidrug and toxic compound extrusion proteins (MATEs), and organic solute transporter (OST). Other than MATEs and OST, the hepatic efflux transport proteins belong to the gene superfamily of ATP-binding cassette (ABC), and they need ATP to provide energy to support their functions.

6.2.1.2.1 MDRs

Within this family, MDR1, also known as P-glycoprotein (P-gp), is the first identified ABC transporter (Chen et al. 1986) and the most well-known canalicular transporter (Gottesman MM 2006). Recently, many variants of MDR1 have been found which arise much attention (Wolking et al. 2015). MDR1 mediates the transport of numerous hydrophobic cations into the bile (Gottesman MM 1993, Gatlik-Landwojtowicz et al. 2006). It also pumps potential toxic substances, including anticancer agents and antivirals, out of the hepatocytes (Sakaeda 2005, Ieiri 2012). The impact of MDR1 affects a lot of drug pharmacokinetics and efficacy (Wolking et al. 2015). In HCC patients, the expression and mRNA level of MDR1 were significantly higher than the normal one (Gao et al. 2015), exacerbating the resistance to anticancer agents. Many attempts have been made to increase the accumulation of anticancer drugs in cancer cells by inhibiting MDR1. Unfortunately, to date, none of these strategies can be applied in the clinic because of drug-drug interactions (DDI) and the increased toxicity issues (Callaghan et al. 2014). For example, a soft tissue sarcoma patient was administered intravenous cyclosporine, which can suppress MDR1 and take VP16/Ifos and VAC cycles as cancer chemotherapy. It was found that cyclosporine markedly elevated the serum concentration of VAC and unwanted toxicity (Theis 1998).

The function of MDR3 is very different from MDR1. As the apical efflux transporter in the liver, MDR3 can translocate phosphatidylcholine (PC) from the

inner to the outer leaflet of the canalicular membrane (Oude Elferink and Beuers 2011). And then PC is released from the outer leaflet into the canaliculus along with bile salts (secreted by BSEP) and forms mixed phosphatidylcholine-bile salt micelles, which is an essential event for the protection of biliary tree from bile acid toxicity (Small 2003).

6.2.1.2.2 BCRP

BCRP is expressed in the canalicular membrane of hepatocytes (Krishnamurthy 2006), and it has a large range of substrates, which partially overlap with those of P-gp and MRPs (Mao and Unadkat 2015), including many endogenous compounds and several therapeutic drugs, such as antibiotics, antivirals, and anticancer drugs (Noguchi and Sugimoto 2014, Mao 2015). A lot of phase II conjugates are included, such as the sulfated conjugates of steroids and xenobiotics (Tian et al. 2015).

By pumping drugs out into the bile, BCRP can undermine the efficacy of clinical drugs (Allikmets et al. 1998). Moreover, the level of BCRP expression may change by disease. For example, its expression in the liver may increase in cancer and decrease by type I diabetes (He et al. 2014, Mao 2015).

6.2.1.2.3 MRPs

Currently, there are nine members of MRP family, which transport a wide range of endobiotics and xenobiotics. Except for MRP2 localized in the canalicular membrane of hepatocytes, MRP families distribute in the basolateral membrane (Gu and Manautou 2010). MRP2 is responsible for the biliary excretion of a wide range of glutathione, sulfate, and glucuronide conjugated endobiotics and xenobiotics, driving bile formation and flow. Its expression is the highest (Wang et al. 2015), suggesting its significant role in xenobiotic disposition and elimination in the liver.

MRP1, MRP3, and MRP4 share parts of the substrates with MRP2 (Evers et al. 2000, Soroka et al. 2001a, Chen ZS et al. 2002), and their expressions are all very low in the liver (Gu and Manautou 2010). If MRP2 is knocked out or deficient, levels of MRP3 and MRP4 may be upregulated to compensate for the lack in biliary excretion of organic anion (Chen et al. 2005, Borst and Van De Wetering 2007). However, the compensation is not complete and may be resulted in accumulation of toxic compounds in the blood or/and liver. Dubin-Johnson syndrome is a genetic basis disease which is associated with conjugated hyperbilirubinemia due to the deficiency of MRP2 (Taniguchi et al. 1996).

MRP1 is not a major transporter in the liver, but it is recently considered to be the most relevant of the MRPs in respect of transport clinical drugs, which becomes a major obstacle in cancer chemotherapy (Leslie et al. 2005). Different from MRP2 and MRP1, MRP3 has very low affinity and capacity to transport GSH

(Kool et al. 1999) and transports natural products without the presence of GSH (Zelcer et al. 2001). MRP4 has the special ability to transport the cyclicnucleotides and nucleotide analogs like adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) (Reid et al. 2003), suggesting that MRP4 may be involved in physiological regulation pathways.

Similar to MRP4, MRP5 has the transport facility for cyclic nucleotide or nucleotide analogs, but its physiological function is still unknown (Borst and Van De Wetering 2007). MRP6 is postulated to be a glutathione conjugate anion pump and contributes to the resistance to certain anticancer drugs in some extent (Belinsky MG et al. 2002). However, its substrates and the role in multidrug resistance await further investigation (Bergen AA et al. 2007).

MRP7 and MRP8 are currently identified to be members of the MRP family. They are both lipophilic anion pumps, which transport a wide range of lipophilic anions, including the glucuronide E217βG, steroid sulfates, glutathione conjugates, and several natural product anticancer agents (Kruh et al. 2007). In addition, MRP8 is highly homologous with MRP5, so it is capable to transport purine and pyrimidine nucleotide analogs such as cAMP and cGMP (Guo et al. 2003). MRP9 is also a new member of the MRP family, and its localization and functional characteristics are unknown until now (Zhang et al. 2015).

6.2.1.2.4 BSEP

BSEP is the primary bile acid exporter on hepatocyte canaliculi (Gerloff et al. 1998). The sequence and structure of BSEP are highly similar to those of P-gp's, so BSEP used to be called sister of P-gp or Spgp (Childs et al. 1995). However, the major function of BSEP is the excretion of conjugated and unconjugated bile salts into the canalicular space (Telbisz and Homolya 2016). Only a few drugs are reported to be the substrates of BSEP, such as pravastatin and fexofenadine (Hirano et al. 2005, Soichiro Matsushima et al. 2008). BSEP is critical in keeping bile salt homeostasis. The impaired function of BSEP is associated with potential DILI. If BSEP is inhibited, no other transporters can compensate completely for bile salt excretion, which is the major reason for in intrahepatic cholestasis and liver injury (Telbisz and Homolya 2016).

Due to the potential importance of BSEP inhibition by drugs, the European Medicines Agency and the International Transporter Consortium have recommended that it should be evaluated in nonclinical safety studies or in human clinical trials (Engevik and Goldenring 2018, Engevik 2018, Anon 2012). Many pharmaceutical companies have included BSEP inhibition assays in their drug discovery and screen programs (Cheng et al. 2016b). But Rodrigues et al. proposed that people should take other mechanisms (downregulation of mRNA/protein expression, etc.) into consideration instead of testing direct inhibition of BSEP alone when screen compounds in a discovery stage (Rodrigues et al. 2014).

6.2.1.2.5 MATEs

Different from other hepatic efflux transporters, MATEs belong to the gene superfamily of SLC. Instead of hydrolyzing ATP during the process of transport, MATEs are coupled with an electrochemical gradient of H+ across membranes (Otsuka et al. 2005). Though MATEs comprise many members, only MATE1 is localized in the liver (Yonezawa and Ken-Ichi 2011), keeping homeostasis by mediating the excretion of metabolic waste products and xenobiotics into bile (Moriyama et al. 2008). MATE1 can export various substrates, which comprises of cationic character, weak bases with positive charge, zwitterions, and part of anions (Staud et al. 2013). Of them, cationic ones are in the majority. There is a substrate overlap between OCT, MATE, and P-gp. OCT takes up these substrates in the blood, while MATE and P-gp excrete them into bile (Moriyama et al. 2008).

6.2.1.2.6 OST

OST is neither sodium-dependent nor ATP-consumed (Dawson et al. 2010) but a facilitated transporter which translocates organic solutes in either direction, just depending on the substrate electrochemical gradient (Ballatori et al. 2005). Expressed in the basolateral membrane in the form of OSTα-OSTβ, OST is responsible for the export of organic solutes including glycine and taurine conjugates of cholic acid, deoxycholic acid, chenodeoxycholic acid, and ursodeoxycholic acid into the blood (Dawson et al. 2010). Compared with BSEP and MRP2, which are the main transporters removing bile acids and other toxic products into the bile, OSTα-OSTβ is expressed at a much lower level and is just an alternative efflux transporter (Soroka et al. 2010). However, in the condition of cholestasis, when canalicular secretion is hindered, OSTα-OSTβ, MRP3, and MRP4 are upregulated to prevent accumulation of hydrophobic bile salts and other toxic products in hepatocytes, acting a hepatoprotective role (Boyer Jl et al. 2006).

6.2.2 The Interspecies Differences of Hepatic Transporters in Abundance and Variety

Hepatic transporters exist distinct interspecies differences both in variety and abundance, which can lead to different hepatic drug concentrations (Lai 2009). Thus, before a drug candidate approved to initiate clinical studies, it is required by the Food and Drug Administration to conduct in vivo toxicity studies in at least two animal species, and one of them must be a nonrodent (Wang et al. 2015).

Compared to human's hepatic OATPs, which contain OATP1A2, OATP1B1, OATP1B3, and OATP2B1, those of rodents are comprised of Oatp1a1, Oatp1a4, Oatp1b2, and Oatp2b1, and those of dogs are Oatp1a2, Oatp1b4, and Oatp2b1. In

addition, there are evident differences between human and rodents in MDR variety. MDR1 and MDR3 expressed in human liver, while Mdr1a, Mdr1b, and Mdr2 are located in the liver of rodents.

In aspect of abundance, the expression of transporters has been determined by quantifying the level of mRNA or protein via quantitative polymerase chain reaction or Western blotting, respectively (Wang et al. 2015). However, the mRNA expressions of transporters are not always correlate with protein level (Ohtsuki et al. 2012). For example, the mRNA level of OAT2 is the highest in the liver compared to other tissues (Fork et al. 2011), but not in proportion to its high mRNA expression; the protein expression of OAT2 is low in human liver (Ohtsuki et al. 2012, Vildhede et al. 2015). Notably, OAT2 has two splice variants, OAT2-546aa and OAT2-548aa. The former is localized to the basolateral membrane, mediating the transport of a broad range of substrates, while the latter is expressed in the intracellular compartment, losing function of cellular uptaking (Cropp CD et al. 2008). In addition, the mRNA and protein levels respond differently when exposed to drugs. For example, when treating Sprague-Dawley rat with pregnenolone 16a-carbonitrile, Mrp2 mRNA levels in the liver are nearly unchanged, while protein levels increase markedly in a time-dependent manner by a gender-independent posttranscriptional mechanism (Johnson Dr and Klaassen 2002).

On the other hand, Western blotting is semiquantitative and needs pure protein standards to compare with. Wang et al. employed a new method to quantify transporter proteins in total membrane protein by using trypsin to digest target transporter proteins and picking two unique signature peptides used as internal standards for liquid chromatography-mass spectrometry (LC-MS/MS). They quantified the protein concentration of BSEP, BCRP, MATE1, MDR1, MRP2, MRP3, MRP4, NTCP, OCT1, and OATPs in human liver and their counterparts in other species (Fig. 6.2). Of them, the expressions of MRP4/Mrp4 and BCRP/Bcrp are

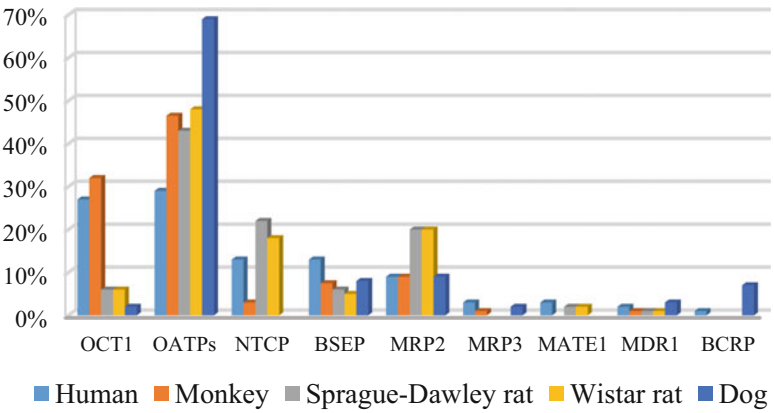

Fig. 6.2 Relative abundance of quantifiable transporters in liver tissue of humans, beagle dogs, cynomolgus monkeys, Sprague-Dawley rats, and Wistar rats (Wang et al. 2015)

difficult to be measured because of their low expression in the liver and relatively high lower limit of quantification (LLOQ) of the signature peptides.

Notably, OATPs, OCT1, and NTCP were the top three abundant influx transporters in human liver. Though the OATPs/Oatps are in the majority of transporters in all these five species, the expression of OATPs in human is relatively lower than those of other species. The expression of OCT1 in human is similar to that in monkeys but is markedly higher than those in rats and dogs. In terms of NTCP, human showed the lower expression than monkeys and the higher expression than rats, while the Ntcp in dogs was not quantified because signature peptides with desired MS sensitivity were failed to be found.

BSEP and MRP2 comprised the most abundant canalicular efflux transporters. The expression of MRP2 was the highest in rats, and those in human, monkeys, and dogs were almost the same, while human showed slightly higher expression in BSEP compared with the other four species. It was reported that agents which did not cause liver injury in preclinical animal investigations displayed liver injury in human by inhibiting BSEP (Stieger 2010a). The difference of BSEP abundance between species may account for this phenomenon. In addition, the expression of OST-α-OSTβ in human liver was reported to be much higher than those in rats and mice (Soroka et al. 2010).

The interspecies differences of hepatic transporters are potential risks of unexpected liver injury in clinical research and become a concern for the pharmaceutic industry. Understanding the interspecies discrepancy instructs researchers to choose appropriate preclinical animal models to assess the effects of drugs and drug candidates on specific transporters and interpret or extrapolate the results from animals to humans (Wang et al. 2015, Cheng et al. 2016b).

In respect to the transporters which are unfeasible to use animal model to predict function because of species differences, like BSEP, several humans originated in vitro models for transporter-mediated liver injury were established and widely used in pharmaceutical industry (Kenna 2014), including membrane vesicle assays, transfected polarized epithelial cells, and isolated hepatocytes for sandwich-cultured hepatocytes.

6.2.3 The Traffic Process of Membrane Transporters in Liver

In the normal condition, the transporters were transferred from cytoplasm to the membrane of hepatocytes after transcription, translation, and posttranslational modification, so the three processes can affect the expression of transporters (Gu and Manautou 2010). Many intracellular components, such as ATP, Ca^{2+}, cytoplasmic motors, and other unknown factors, are reported to be involved in transporter trafficking to the surface of hepatocytes (Kipp and Arias 2000).

Although the transporters are localized in the membrane of hepatocytes, some drugs and disease can redistribute them by affecting transcription, translation, and posttranslational modification, leading to the changes in the expression and function

of transporters. For instance, inchinkoto can significantly increase the expression levels of MRP2/Mrp2 by posttranscriptional modulation (Watanabe et al. 2009a). In rat liver, hyperosmolarity induces a retrieval of the Bsep and Mrp2 from the canalicular membrane by activating the Src family kinase Fyn, resulting in cholestasis (Miriam Cantore et al. 2011). In contrast, choleretic agents, such as cAMP and tauroursodeoxycholate (TUDC), trigger insertion of the hyperosmotically retrieved transporters into the cell surface, making up the shortage of Bsep and Mrp2 in the membrane (Kurz et al. 2001).

The redistribution of membrane transporters in the liver may account for the inconsistence of transporter protein level with transporter functions when in response to oxidative stress, for the transcriptional and translational regulations are much slower than the trafficking of transporters. So it's not always a good way to analyze the function of transporters by protein expression levels. It's wise to verify whether the activity of the transporters is proportional to its expression level before quantification.

6.3 Roles of Hepatic Transporters in Drug Disposition, Metabolism, and Elimination

In the previous section, the category and location, transport characteristics, substrate and inhibitor/inducer profiles, and species differences of hepatic transporters had been described. In this section, we will systematically address the effects of hepatic transporters on drug distribution, metabolism, and excretion, which collectively refer to as drug disposition.

The liver is the first organ to be in contact with the absorbed drugs after oral administration and portal vein transport. It is the most important xenobiotic detoxification organ and has a decisive responsibility for xenobiotic exposure. The hepatic transporter system is an indispensable part of the complex xenobiotic elimination system. As mentioned in the previous section, a large number of drugs are substrates of hepatic uptake/efflux transporters. Changes in the function of these transporters can significantly affect the drug disposition, which finally may affect the toxicity/efficacy of these drugs.

The great influence of transporters in liver clearance can be indicated in the empirical hepatic clearance equation. The hepatic clearance ($CL_{H,int}$) can be described by the following equation (Yamazaki et al. 1996):

$$CL_{H,int} = \frac{PS_{influx}\left(CL_{H,met} + CL_{Biliary}\right)}{PS_{efflux} + \left(CL_{H,met} + CL_{Biliary}\right)}$$

Here, $CL_{H,int}$, $CL_{H,met}$, and $CL_{Biliary}$ represent the overall hepatic intrinsic clearance, hepatic metabolism clearance, and biliary excretion; PS_{influx} and PS_{efflux} are the apparent membrane transport clearance.

In addition, we will also briefly describe the effect of efflux transporters on liver non-parenchymal cells (hepatic stellate cells, hepatic proliferative stem cells, etc.) on drug disposition and physiological function of these cells in a variety of hepatic physiologic/pathological states.

6.3.1 Hepatic Transporters and Drug Distribution

For drugs with moderately lipophilic and permeable to cell membrane, passive diffusion is the major route to enter the liver (Sugano et al. 2010). Even if these drugs also are substrates for transporters, the contribution of active transport may be negligible (Cao et al. 2005). This type of drug disposition, which is dominated by passive diffusion, is also called perfusion-limited distribution.

For these drugs have relatively poor solubility and membrane permeability (especially BDDCS 3 and 4 drugs), the liver transporter system (particularly uptake transporters) plays essential roles in drug distribution. In some cases, these uptake transporters dominate drug disposition process.

For example, Sugiyama indicated that although multiple metabolic enzymes (mainly CYP enzymes) and transporters (both uptake and efflux transporters) mediate the disposition of statins, OATP is the central determinant (Maeda et al. 2011). It was estimated that if the activity of uptake transporters (OATPs) decreased to 1/3 of normal, pravastatin blood concentration can be increased nearly three times. While if the uptake transporter activities increased threefold, pravastatin's plasma concentration will be reduced to 14% of the normal values. CYP enzymes and efflux transporters, which mediate the metabolism and biliary excretion of statin, do not cause significant changes (Watanabe et al. 2009b). The liver-specific distribution of pravastatin via OATP maintains high concentration and pharmacological effect in the liver while a relatively low plasma concentration, which means lower side effects in other tissues. In clinic, increase plasma exposure of statins may lead to rhabdomyolysis toxicity. It is not surprise that co-administration of OATP inhibitors (such as gemfibrozil) can result in a 1.5- to 2.0-fold increase in the mean AUC of statins (pravastatin, pitavastatin, and rosuvastatin), which increase the risk of myopathy (Backman et al. 2000, Whitfield et al. 2011).

Another example to revealing hepatic uptake transporters on drug disposition and efficacy/toxicity is berberine, one of the most commonly used natural compounds. Berberine exhibits a low plasma concentration both in human and rats: the maximum concentration (C_{max}) of berberine in human plasma was measured at 0.4 ng/ml after a single oral dose of 400 mg (Hua et al. 2007) and 4 ng/ml after oral administration of 100 mg/kg in rats. Interestingly, the concentration of berberine is more than 1600 ng/g tissue in rat liver, which was approximately 70-fold greater than that in plasma (Liu et al. 2009, Liu et al. 2010). One of the important reasons for this is that berberine is a substrate for both OATP and OCT (Kwon et al. 2015).

NTCP plays critical role in hepatitis B virus (HBV) infection or "HBV distribution" (Yan et al. 2012). NTCP was identified as an HBV entry receptor, and studies have

shown that NTCP knockout or inhibition can significantly reduce or even eliminate the invasion of hepatitis B virus (Nakabori et al. 2016). Those results revealed the potential physiological functions of NTCP and other uptake transporters.

The changes of transporter expression have impacts on the disposition and efficacy of drugs. In renal cell carcinoma, DNA hypermethylation inhibits H3K4trimethylation at the OCT2 promoter leading to OCT2 repression, which led little oxaliplatin (substrates of OCT2) uptake into cell, resulting in drug resistance (Liu et al. 2016). For decades, people worked hard to tackle transporter-related drug resistance in cancer cells. For example, MRP2 mRNA and protein expression level significantly increased in hepatocellular carcinoma cell (HCC) compared to nonneoplastic liver tissues (Bonin et al. 2002, Zollner et al. 2005). MRP2-related loss in therapy efficacy has been described for chlorambucil (Smitherman et al. 2004), doxorubicin (Kool et al. 1997), cisplatin (Korita et al. 2010), etc. It was proposed that the MRP2 inhibitors MK-571 or cyclosporin A may increase antitumor therapeutic effectiveness (Leier et al. 1994, Chen et al. 1999). However, the poor specificity of these inhibitors may increase risk of untargeted toxicity in clinic (Materna et al. 2005).

6.3.2 *Hepatic Transporters and Biliary Excretion*

Biliary excretion is one of the three most important xenobiotic excretion routes (Urinary excretion and Pulmonary excretion), which highly depends on the efflux transporters along canalicular membranes of hepatocytes.

In addition to passive diffusion, the uptake transporters on the hepatocyte sinusoid side facilitate the transport of the substrates into the liver cells; and the efflux transporters on the canalicular side or the sinusoidal side are responsible for the transport of parent drugs and their metabolites to the bile or back to the blood by MRP3/4. Transporters (P-gp, BCRP, BSEP, etc.) are critical in the clearance of hepatic elimination in the body. For instance, intravenous administration of imatinib in Mdr1a/1b/Bcrp1$^{-/-}$mice, the biliary excretion of imatinib was significantly reduced 8.1-fold compared with wild type (Oostendorp et al. 2009). Nitrofurantoin, an antibiotic drug, was found to be mainly eliminated by biliary excretion contributed to Bcrp. And hepatobiliary excretion of nitrofurantoin was almost abolished in Bcrp knockout mice (9.6% in wild type while 0.2% Bcrp1 in knockout mice) (Merino et al. 2005).

Drug biliary excretion is highly dependent on enterohepatic circulation of endogenous bile acids. Primary bile acids (cholic acid (CA) and chenodeoxycholic acid (CDCA)) are synthesized from cholesterol via CYP7A1 and CYP8B1 or alternatively via CYP27A1 in the liver and are important regulators of physiological processes such as cholesterol catabolism (Russell 2003). They are excreted into the bile duct lumen via BSEP and MRP2 on hepatocytes (Kullak-Ublick et al. 2004). The active transporters of phospholipids (via MDR3), cholesterol (via ABCG5/8), and glutathione (also via MRP2) are also involved (Thomas et al. 2008, Wagner

et al. 2009). Across the bile ducts, BAs are released into the upper small intestine (duodenum). Then, BAs may be reabsorbed by active uptake into enterocytes via apical sodium-dependent bile acid transporter (ASBT) and exported into blood via organic solute transporter (OST) (Thomas et al. 2008, Wagner et al. 2009). While in the gut, the primary bile acids are metabolized by bacteria to form secondary bile acids (CA to deoxycholic acid (DCA), CDCA to lithocholic acid (LCA) and ursodeoxycholic acid (UDCA) (Trauner and Boyer 2003). From the portal circulation, both primary and secondary bile acids are selectively imported into hepatocytes by an active transporting mechanism, mainly consisting of NTCP (high affinity for conjugated bile salts) and OATP (high affinity for unconjugated bile salts) (Weinman 1997). During the enterohepatic recirculation process, 90% of the bile acid pool in the gut is reabsorbed, and only a small fraction is lost in feces and replaced by de novo synthesis in the liver (Trauner and Boyer 2003, Ridlon et al. 2006). Drug biliary excretion shared MRP2 with bile acid excretion, and BCRP, MATE, P-gp are also involved (Kock and Brouwer 2012).

It is worthwhile to note that some drugs also utilize very close enterohepatic circulation. For example, saponins, immunosuppressive drugs (e.g. mycophenolic acid) and some antibiotics (e.g. ampicillin), after being catalyzed by glucuronic acid transfer enzyme, will be transformed into glucuronide conjugates, which are secreted by MRP2 within bile into the intestine. And the bacteria in the intestine may hydrolyze the conjugates into prototype drug, which will be reabsorbed (Ieiri et al. 2009).

6.3.3 Transporter-Transporter Interplay

As mentioned in the previous section, many drugs are substrates of uptake/efflux transporters, and some are substrates of multiple uptake/efflux transporters. For example, methotrexate is a substrate of up to 26 transporters (Mikkelsen et al. 2011). Almost all the known efflux transporters can pump MTX out of hepatocytes. Therefore, the loss of function of a single efflux transporter will not have significant impacts on MTX disposition (Vlaming et al. 2009a). It was found that double or triple knockouts of efflux transporters were required to influence MTX disposition (Vlaming et al. 2009c). This compensatory effect minimizes drug disposition fluctuation and toxicity risk in spite of specific efflux transporter dysfunction. In several pathological conditions, the expression and function of the related transporters may be upregulated if their regular activities cannot make up for the impaired transporter capacity. For example, the downregulation of Mrp2 may lead to the upregulation of Mrp3/4 in cholestasis (Soroka et al. 2001b). In the Bcrp/Mrp2 double knockout ($Bcrp^{-/-}/Mrp2^{-/-}$) mouse models, the function of MRP3 was upregulated in a compensatory way (Vlaming et al. 2009b). These phenomena were not only found in hepatocytes, and similar events were also found in the brain. For instance, both P-gp and Bcrp can reduce the brain penetration of erlotinib. However it was found that concentration of erolotinib in the brain of double knockout (mice was higher than

P-gp$^{-/-}$ or Bcrp$^{-/-}$ single knockout ones (Geyer et al. 2009), which proved the co-regulation and compensation between the two transporters in erlotinib distribution. Such compensatory effects may increase drug toxicity/efficacy and meanwhile the proportion of renal clearance.

Although the compensatory mechanisms of transporters in pathological conditions are not thoroughly studied, nuclear receptors may be important regulatory factors. For example, Nrf2, Pxr, and Ahr activations have been reported to upregulate a variety of transporters (Mrp2/3/4, P-gp and Bcrp, etc.) (Gu and Manautou 2010, Chan et al. 2013).

6.3.4 Transporter-Metabolism Interactions

Liver is the major site of drug metabolism, accounting for approximately 70% of drug elimination in humans. Drug-metabolizing enzymes convert substances into their pharmacologically inactive form, and the most well-known drug-metabolizing enzymes are cytochrome P450s (CYP450s). The contribution of both drug-metabolizing enzymes and drug transporters is important in metabolism, for many drugs need to be taken into cells by uptake transporters before being metabolized, and their metabolites can be pumped out hepatocytes by efflux transporters.

Drugs must enter the cell to be metabolized by the metabolic enzymes in the cell. In a lot of cases, transporter-metabolizing enzyme interactions are valuable to drug's clearance. Their joint presence in hepatocytes, by the significant overlap in their substrate specificities, increases metabolic efficacy.

In the intestine, the CYP3A-mediated metabolism and P-glycoprotein-mediated counter-transport in the enterocyte increased the mean residence time of drug molecule in enterocytes, allowing CYP3A to have repeated access to these molecules (Zhang and Benet 2001). In other words, the overlaps in the substrates between CYP3A and P-gp increase the access of drug for metabolism by CYP3A through repeated cycles of absorption and efflux (Zhang and Benet 2001). In the liver, CYP3A4 continues to metabolize drugs, and P-gp pumps these drugs and their metabolites out through biliary excretion.

In the liver, drugs are metabolized to nontoxic/less toxic metabolites. This metabolism process usually increases hydrophilicity of drugs and makes them not easy to passively move out of the cell membrane. Subsequently, efflux transporters may recognize the conjugates of metabolites (GSH, sulfated groups, etc.) and pump them out hepatocytes. For example, APAP is not a substrate of efflux transporters, but its phase II metabolites are substrates of Mrps. The generation and efflux of nontoxic phase II metabolites increase the ratio of APAP conversion, thereby reducing the hepatotoxicity of APAP (Manautou et al. 2005).

An opposite example is some drugs that cause iDILI in the clinic are focused on the damage of biliary epithelial cells. Studies have found that the accumulation of these toxic metabolites (GSH conjugates) in hepatocytes after Mrp2 knockout

decreased amount of these drugs in biliary epithelial cells and weakened liver toxicity (Dietrich et al. 2001).

Actually, the relationship between transporters and drug enzyme is much more complicated. It was noted that P-gp andCYP3A have large overlap in substrates and common inducers (e.g., rifampin, phenobarbital, dexamethasone) (Wacher et al. 1995). When drugs enter into the hepatocyte, they are metabolized by the CYP3A; and the metabolites are transported out into blood or the lumen; molecules that escape metabolism are eliminated from the cells via P-gp and other efflux transporters. Drugs inhibit P-gp may also inhibit CYP3A4; and the dual inhibition effects will increase drug exposure. For instance, cyclosporine A can inhibit both P-gp and CYP3A4 function. Cyclosporine A administration could significantly increase the bioavailability and efficacy of paclitaxel (Kuppens et al. 2005).

6.3.5 Transporters in Non-parenchymal Liver Cells

The above sections have focused on the effects of transporters on parenchymal liver cells (hepatocytes). However, it has been found that drug transporters, efflux transporters in particular, have unique expressions and functions on these non-parenchymal liver cells as well. And these transporters have significant impact on liver pathology under specific conditions.

For example, Mrp1 is highly expressed and functional in activated hepatic stellate cells (HSC) during liver fibrosis, which may increase the survival possibility of activated HSC in the pathological state of liver fibrosis (Hannivoort et al. 2008). Actually, spontaneous apoptosis of HSC occurred when Mrps were inhibited using MK-571 (broad-spectrum inhibitor of Mrps) (Hannivoort et al. 2008). Given that activated HSC are central regulator of liver fibrosis, the inhibition of Mrps is likely to be a target for the treatment of hepatic fibrosis. There is report that the MRP1 inhibition in CCl_4-induced liver fibrotic mice led to the suppressing of HSC activation through the Hedgehog pathway (Sun et al. 2017).

Hepatic progenitor cells play key role in regeneration and repair of liver after liver damages (Kaur et al. 2015). It was found that under a variety of pathological conditions (such as hepatic injury and regeneration caused by liver resection; LPS-induced liver inflammation), Mdr1b expression dramatically increased (up to 50–100-folds), whereas other efflux transporters barely changed in hepatic progenitor cells (Vos et al. 1999, Ros et al. 2003, Mei et al. 2004, Nishimura et al. 2005, Vos et al. 2006). Mdr1b is a subtype of P-gp. Its contribution in drug elimination is negligible when compared with its counterpart, Mdr1a. For example, our study indicated that Mdr1b knockout has almost no effect on the disposition of berberine, a well-known P-gp substrate (unpublished data). At present, whether the significant upregulation of Mdr1b is only a sign of synchronous stress response or indeed has essential role in liver regeneration is still unclear, and further investigation is needed.

In conclusion, efflux transporters expressed on those non-parenchymal liver cells likely to affect important cellular functions (cell survival, cell proliferation, etc.).

6.4 Inter-individual and Inter-ethnic Variability in Drug Metabolism and Disposition: Liver Transporter Polymorphisms

Transporters have multiple single nucleotide polymorphisms (SNPs), and some SNPs are associated with the activity of the transporter, which affects the pharmacokinetics and changes the exposures of the drugs.

6.4.1 OATP Polymorphism

OATPs are a class of important drug uptake transporters, which are highly correlated with the ADME of drugs. OATP1B1 polymorphisms have gained considerable attention due to their significant impacts on a wide range of drug disposition. Tirona and coworkers identified 14 nonsynonymous genetic variations of OATP1B1 in European- and African-Americans (Tirona et al. 2001). Nozawa et al. (2002) found that the OATP1B1∗1b (A388G) and ∗5 (T521C) alleles were present with 54% and 0.7% in the Japanese population, respectively. Now more than 400 mutations were found in human.

Among them, OATP1B1 T521C and A388G polymorphisms were reported in multiple clinical studies. The variant T521C mutation (OATP1B1∗5) may decrease OATP1B1 activity and uptake function, limiting entry of drugs into liver and increasing drug plasma concentrations. It has been reported that the T521C mutation reduced hepatic clearance of lopinavir and multiple statins, which include pravastatin, rosuvastatin, atorvastatin, and simvastatin acid (Ho et al. 2007, Hartkoorn et al. 2010, He et al. 2015a) (Pasanen et al. 2007, Birmingham et al. 2015a, b).

OATP1B3 has 80% amino acid identity to OATP1B1 (König et al. 2000), and they have a lot of overlapping substrates, like statins and antibiotics. Letschert et al. firstly identified additional mutations T334G (OATP1B3p.S112A) and G699A (OATP1B3p.M233I) in the Caucasian population (Letschert et al. 2004). It was reported that effects of the T334G and G699A did not lead to significant transport substrates of OATP, like BSP and E217βG (Letschert et al. 2004), and it was also found that the two mutations wouldn't lead to significant change in clearance of total paclitaxel in patients (Smith et al. 2007).

6.4.2 P-gp Polymorphism

P-gp is usually regarded as the most important efflux transporter. The first genetic variants of P-glycoprotein, T103C and G137 T, were found in the P-gp promoter region of human osteosarcoma cells in 1994 (Stein et al. 1994). After that more and more SNPs have been identified in the coding region, but many of these variants just

have putatively functional impacts. For example, SNP G1199 T is associated with lower transport capacity in vitro, which leads to putatively higher sensitivity against cytostatics (Crouthamel et al. 2006). Studies about P-gp SNPs mainly involve the G1199A in exon11, C1236T in exon12, G2677 T/A in exon21, and C3435T in exon26. C3435T is the first identified silent polymorphism. The functional significance of the P-gp variants is still under discussion. It was reported that C3435T mutation may impair mRNA secondary structure, associated with lower mRNA and protein levels in human liver (Wang et al. 2005, Cascorbi 2006). It was found that the plasma concentration of digoxin was significantly higher in patients with C3435T variants (Hoffmeyer et al. 2000).

6.4.3 BCRP Polymorphism

At this time, more than 400SNPs in human BCRP coding region have been reported (Honjo et al. 2002, Iida et al. 2002, Imai et al. 2002, Backstrom et al. 2003, Itoda et al. 2003, Bosch et al. 2005, Kobayashi et al. 2005a, b, Lee et al. 2007). There are two frequently polymorphic SNPs in the BCRP gene: G34A in exon2 andC421A in exon5.

The polymorphism of BCRP C421A results in glycine-to-lysine amino acid change and has higher frequencies in Asians than European- or African-Americans (Zamber et al. 2003). This particular SNP was linked to lower membrane expression (Imai et al. 2002), transporter function (Morisaki et al. 2005, Lee et al. 2007), reduced ATPase activity (Mizuarai and Aozasa 2004), and substrate specificity in vitro (Tamura et al. 2007).

The clinical impact of the frequent occurring BCRP polymorphisms (C421A and G34A) was studied by many researches. It was observed in 156 Asian patients treating with a BCRP substrate, irinotecan, that patients carrying G34A variant showed high rate of diarrhea and lower AUC of SN-38, a metabolites of irinotecan (Han et al. 2009). The phenomenon implied that G34A variants may be less efficient at pumping irinotecan and its metabolites out of cells which are associated with the higher occurrence of diarrhea.

The effects of BCRP polymorphisms (C421A) are a bit of controversial. For instance, it was found that there is no significant effect of the C421ASNPs on pharmacokinetic parameters of irinotecan (de Jong et al. 2004). And this polymorphism does not affect plasma concentration of nitrofurantoin (Adkison et al. 2008). But it also has been reported that the C421A SNP results in a higher gefitinib accumulation in patients (Li et al. 2007). Patients with BCRP C421A had a higher rate of diarrhea than those who lack it. Similarly, oral rosuvastatin exposure was approximately twofold higher in C421A variant patients than patients with the wild type (Zhang et al. 2006b). Keskitalo et al. also detected higher plasma concentrations of fluvastatin and simvastatin in patients with BCRP C421A variants. For BCRP C421A variants may be caused by the low expression of transporter (Imai et al. 2002). It is speculated that the C421A SNP may affect BCRP cellular stability and enhance microRNA-dependent BCRP repression (Ripperger and Benndorf 2016).

6.4.4 MRP Polymorphism

MRPs may have the most abundant SNP variants in drug transporters: thousands of SNP variants have been listed in the NCBI SNP database. Whereas most sequence variants have no phenotypic consequences, some may impair transporter's expressions and functions and even cause disorders or disease, for example, Dubin-Johnson syndrome (MRP2), pseudoxanthoma elasticum (MRP6), and altered earwax and osmidrosis syndrome (MRP8).

The most extensively studied genetic variant of MRP transporters is the MRP2 gene. Among the nearly one thousand MRP2 SNP variants, the impacts of four genetic polymorphisms (C24T, G1549A, A1019G, and C3972T) have been extensively studied. The combination of the G1549A and A1019G variants is common in Caucasians and African-Americans (Tan et al. 2012). It was found the G1549A and C-24 T decreased MRP2 promoter activity (Choi et al. 2007). The C3972T had a significant effect on the tacrolimus and irinotecan concentration in patients (Ogasawara et al. 2013). The C-24 T polymorphism effects on MRP2 expression are controversial (Moriya et al. 2002, Anderson et al. 2006, Naesens et al. 2006, Haenisch et al. 2007).

6.5 Transporter-Mediated Drug Hepatotoxicity

The specific distributions of drug mediated by transporters have both advantages and disadvantages. The tissue-specific distribution of drugs in the liver may enhance their exposure and toxicity as well. They are critical for a lot of drugs' disposition and elimination process in the liver. Therefore, they are highly involved in drug's hepatotoxicity.

6.5.1 Hepatic Transporters and Drug-Induced Liver Injury

According to the original International Consensus Criteria, liver injury due to drugs is classified as cholestatic, hepatocellular, or mixed pattern (Benichou et al. 1993, Danan and Benichou 1993). More and more reports have implicated that hepatic transporters are highly involved in the generation of DILI, the cholestatic DILI in particular (Godoy et al. 2013). In this chapter, we will focus on the role of hepatic transporters in the generation of DILI. Transporter gene polymorphisms and altered transporter expressions and functions due to diseases are found to be susceptible factors for drug-induced liver injury (DILI) (Cheng et al. 2016a).

6.5.1.1 Transporters Mediate the Absorption of Toxic Compounds

Transporters have a wide range of substrates, including endogenous and exogenous substances, and transport them without distinguishing whether they are toxic or not, so it frequently happens that transporters mediate the influx and accumulation of toxic compounds, enhancing their hepatotoxicity.

OCTs have some toxic substrates, ranging from herbal medicine like monocrotaline (MCT) (Tu et al. 2013) and nitidine chloride (NC) (Li et al. 2014) to platinum-based anticancer drug oxaliplatin (Zhang et al. 2006a). Take monocrotaline (MCT) as an example. As a pyrrolizidine alkaloid (PA), MCT is activated by cytochrome P450 (CYP) enzymes in the liver and then induces hepatotoxicity. As a result, the influx of MCT to the liver is the key step for its hepatotoxicity. In vitro study has revealed that OCT1 mediates the hepatic uptake of MCT and may play an important role in MCT-induced hepatotoxicity (Tu et al. 2013).

OATP is another family of transporters which should be blame for toxin absorption. Their toxic substrates include mushroom toxin phalloidin, the blue-green algae toxin microcystin-LR (Lu et al. 2008), phalloidin analog demethylphalloin (Fehrenbach et al. 2003, Meier-Abt et al. 2004), and so on. In the case of phalloidin, which can accumulate in liver cells, blocking actin filaments and leading to hepatocyte necrosis (Wieland 1983), its high-efficiency uptake into hepatocytes is mainly carried out by OATP1B1 and OATP1B3 in human and rat (Meier-Abt et al. 2004).

6.5.1.2 Transporter Polymorphisms Increase the Risk of Drug Toxicity

As described earlier in this article, genetic variations in drug transporters may change drug pharmacokinetics, leading to reduce drug efficacy and increase clinic risk.

For example, OATP1B1 c.521 T > C polymorphism was reported to be associated with toxic side effects caused by the anticancer drugs irinotecan (Han et al. 2008, Sai et al. 2010) and methotrexate (Lopez-Lopez et al. 2011). Also, polymorphism of the genes encoding OATP1B1 and OATP1B3 is associated with increased serum levels of unconjugated and conjugated bilirubin in humans, which may eventually lead to hyperbilirubinemia (Keppler 2014, Lapham et al. 2016).

Polymorphisms in efflux transporters have also raised concerns. It has been reported that genetic mutations in MRP2 can cause an autosomal recessive liver disorder called Dubin-Johnson syndrome (DJS), characterized by chronic conjugated hyperbilirubinemia (Paulusma et al. 1997). Also, loss-of-function mutations in MRP2 were associated with impaired methotrexate elimination and an increased risk of toxicity (Hulot et al. 2005). It had been recommended that candidates for methotrexate therapy should be considered for MRP2 functional testing. In addition, MDR3 mutations and polymorphisms may (pre-)determine individual susceptibility to acquired cholestatic liver injury caused by drugs and inflammatory cytokines (Trauner et al. 2007).

6.5.1.3 BSEP Inhibitors and Cholestasis

Some drugs are inhibitors of transporters, affecting their functions and disrupting the homeostasis of endogenous substrates, which results in liver injury. For example, reduced efficiency or complete inhibition of BSEP can lead to the accumulation of bile salts and other BSEP substrates in the hepatocytes and cause cholestasis and other damages to the liver. Many drugs with potent inhibition of BSEP activity (IC50 < 25 μM) are reported to cause cholestatic and mixed cholestatic and hepatocellular injuries, such as cyclosporine, ritonavir, rosiglitazone, saquinavir, troglitazone, ketoconazole, pioglitazone, lovastatin, haloperidol, atorvastatin, and chlorpromazine (Stieger et al. 2000, Byrne et al. 2002, Zamber et al. 2003, Stieger 2010b, Stieger and Beuers 2011).

It was reported that increased serum total bile acid and conjugated bilirubin concentrations were indicators of cholestasis, which account for approximately 30% of all cases of drug-induced liver injury (Zamek-Gliszczynski et al. 2012). However, despite the large number of drugs showing BSEP inhibition in vitro, some researchers questioned the in vitro-in vivo correlation (IVIVC) of BSEP inhibition assessment by pharmaceutical companies.

That's because all of these in vitro models show some ineluctable limits for the prediction of BSEP inhibition. For example, the most widely used membrane vesicles from transfected insect cells could not detect BSEP inhibition requiring cooperativity with other biliary transporters and not the effect of drug metabolites on BSEP for lack of metabolic activity (Stieger et al. 2000). Cellular assays were limited for their much higher cost and lower throughput capabilities than BSEP expressing vesicle assays (Kenna 2014). Some studies also emphasized that it is unwise to evaluate BSEP inhibition data alone without considering other drug safety issues. Besides, it is unclear whether these effects are relevant to humans at the clinical therapeutic doses. All in all, we may have a better understanding of the relationship between BSEP inhibition and model selection by taking account of mechanisms of cell injury and drug exposure in vivo.

6.5.2 Transporter Inhibitors and Liver Toxicity

Alteration in activities of hepatic transporters due to DDI, genetic polymorphisms, and disease states can result in changes in pharmacokinetics of transporter substrate drugs, which may subsequently influence their pharmacological or toxicological effects. By understanding the mechanism of how drugs interfere with hepatic transporters' function or vice versa, we may find better therapeutic strategies targeting hepatic transporters for DILI. The list of drugs that has been associated with hepatotoxicity is very long. In this part we will only highlight several drugs which cause liver toxicity through transporter inhibitions.

6.5.2.1 Bosentan (Fouassier et al. 2002)

Bosentan is often used for the treatment of pulmonary arterial hypertension (PAH) due to its nonselective inhibiting of endothelin receptor (Seferian and Simonneau 2013). A long-term safety study of bosentan from year 2009 to 2014 had showed that 16.8% of patients receiving bosentan experienced elevations of alanine and/or aspartate aminotransferases beyond the upper limit of normal, which was the main reason for discontinued treatment of PAH with bosentan (Simonneau et al. 2014).

Although the mechanisms underlying bosentan-induced hepatotoxicity have not been fully understood, cholestatic or mixed liver injury is considered to be the major hepatotoxic symptom of bosentan. Bosentan and its active plasma metabolite Ro 48–5033 are uptake by OATP1B1 and OATP1B3 into the hepatocytes, and bosentan can be excreted into bile by MRPs (Treiber et al. 2007). Despite the direct hepatotoxicity caused by bosentan, its reactive metabolite R0-64-1056 metabolized by CYP2C9 also showed hepatotoxicity (Matsunaga et al. 2016).

The cholestatic liver injury of bosentan is mainly due to direct inhibition of BSEP. Although both rat and human Bsep/BSEP are inhibited by bosentan (Fattinger et al. 2001, Dawson et al. 2012), bosentan-induced hepatotoxicity was not observed in rats. These species differences can be explained by the differential inhibition of Ntcp and NTCP between rats and human. Bosentan inhibition of Ntcp-mediated bile acid uptake could counterbalance Bsep inhibition, reducing bile acid accumulation in the liver, thereby preventing hepatotoxicity in the rat. But human NTCP was not potently inhibited by bosentan (Leslie et al. 2007).

Another hypothesis suggested that bosentan altered canalicular bile formation via stimulating the transport activity of MRP2/mrp2/ABCC2 (Fouassier et al. 2002, Mano et al. 2007). If BSEP inhibition is responsible for cholestasis and liver toxicity, the bile salt excretion should be reduced after bosentan treatment. However, no impact of bosentan on bile salt output was observed. On the contrary, biliary phospholipid secretion was reduced in the animal study (Fouassier et al. 2002).

According to the theory of Neil Kaplowitz, the secretion of phospholipids and cholesterol depends upon the multidrug resistance-2 P-glycoprotein (mdr2)(Elferink et al. 1997). Glutathione efflux through the multidrug resistance protein-2 (mrp2) and bicarbonate secretion act as driving forces for bile salt-independent canalicular bile flow (Zhang et al. 2009). Under the condition when mrp2 was activated by bosentan administration, increased canalicular bile flow with constant bile salt output led to a lower intracanalicular bile salt concentration, resulting in a reduction in biliary lipid secretion (Small 2003). Consequently, the lipid asymmetry of the canalicular membrane may be adversely altered and thus in turn lead to the intracellular accumulation of bile salts, leading to the generation of cholestasis (Meier 2002). However, this theory didn't explain the species difference between human and rodents.

6.5.2.2 Troglitazone (Snow and Moseley 2007, Yabuuchi et al. 2008)

Troglitazone (TGZ), a peroxisomal proliferator-activated receptor (PPAR) γ agonist that improved insulin resistance, was used for the treatment of type 2 diabetes (Inzucchi et al. 1998, Taylor 2004). But it was withdrawn from the market by the US Food and Drug Administration (FDA) in 2000 because of its association with acute liver failure (Graham et al. 2003). It was speculated that TGZ or TGZ sulfate (TGZS) induced liver injury by direct mitochondrial damage and oxidative stress (Chojkier 2005). However, another popular theory is TGZ, and its major metabolite TGZS induced hepatotoxicity by BSEP inhibition, which will lead to retention of bile salts in hepatocytes and cause hepatocyte apoptosis (Kwo et al. 1995, Patel et al. 1999). What's more, high intracellular bile salt levels have been reported to induce cell death and mitochondrial dysfunction due to their detergent properties (Delzenne et al. 1992, Gores et al. 1998). Considering that TGZS, a potent inhibitor of bile acid efflux transporters, is extensively excreted into bile via Mrp2 (Kostrubsky et al. 2001) and Bcrp (Enokizono et al. 2007), it is speculated that alteration of Mrp2 or Bcrp due to genetic polymorphism, disease, or drug-drug interaction may account for the accumulation of TGZS in the liver. For example, diabetic patients with a history of cholestasis may decrease the liver capacity to excrete TGZS into the bile and therefore have an increased risk of developing hepatotoxicity (Kostrubsky et al. 2000).

6.5.2.3 Cyclosporine A

Cyclosporine A (CsA) is a potent immune suppressor that is widely used in transplant surgery and the treatment of several autoimmune diseases (Tedesco and Haragsim 2012). However, upon its initial use in the 1970s, CsA was found to be nephrotoxic (Tedesco and Haragsim 2012). Shortly after that, it had been reported that systemic treatment with CsA may result in an impairment of the biliary excretion of bile salts and cholestasis both in human (Gulbis et al. 1988) and in rats (Le Thai et al. 1988, Stone et al. 1988, Roman et al. 1990).

Generally, the mechanisms of CsA-induced cholestasis come down as follows:

(i) Competitive inhibition of ATP-dependent biliary transporters, such as P-gp, BSEP, MRP2, and BCRP (Bohme et al. 1993, Kadmon et al. 1993, Bohme et al. 1994)
(ii) Inhibition of intrahepatic vesicle transport and targeting of ATP-dependent transporters on the canalicular membrane (Roman et al. 1990, Roman and Coleman 1994, Roman et al. 2003)
(iii) Impairment of bile secretion partly by increasing canalicular membrane fluidity without affecting the expression of canalicular transporters (Yasumiba et al. 2001)

6.5.2.4 Statins

Statins have been widely used for the treatment of hypercholesterolemia and atherosclerotic cardiovascular disease (Bjornsson et al. 2012, Kitzmiller et al. 2016). Besides myotoxicity, among the most frequently noted side effects of statin treatment is liver toxicity (Kromer and Moosmann 2009). Up till now, 1.9–5.5% of patients with drug-induced liver injury have been reported to occur due to statins, especially atorvastatin and simvastatin (Bjornsson 2017).

A great number of studies have indicated that transporters play a key role in statin pharmacokinetics which may have influence on statin efficacy and toxicity (Russo et al. 2009). The most often mentioned is the genetic polymorphism in SLCO1B1 (Kitzmiller et al. 2016) and ABCG2 (Keskitalo et al. 2009), encoding the statin uptake transporter OATP1B1 and efflux transporter BCRP. Individuals carrying a dysfunctional genetic variant in SLCO1B1 can lead to impaired hepatocellular uptake of statins, resulting in their significantly elevated systemic exposure, which may explain statin-induced liver injury (Sadighara et al. 2017).

In addition to transporter polymorphism, drug-drug interactions can also alter the function of OATPs (mainly OATP1B1) and BCRP (Hirota and Ieiri 2015). Multiple studies demonstrated that the blood exposure of statins can be increased by OATP inhibitors, like rifampin (Lau et al. 2007, Maeda et al. 2011, Chang et al. 2014), gemfibrozil (Karlgren et al. 2012), cyclosporine (Shitara et al. 2003), and so on.

6.5.2.5 TAK-875

Fasiglifam (TAK-875), a G-protein-coupled receptor 40 (GPR40) agonist in development for the treatment of type 2 diabetes, was terminated in phase 3 due to adverse liver effects (Kaku et al. 2015, 2016). Several researches have been trying to identify the risk factors for TAK-875 hepatotoxicity, which is important for the design of the next generation of GPR40 agonists. According to the research findings, formation of TAK-875AG, and possibly TAK-875CoA in hepatocytes, coupled with inhibition of hepatic transporters and mitochondrial respiration may be key contributors to TAK-875-mediated DILI (Li et al. 2015, Otieno et al. 2017b, Wolenski et al. 2017).

For example, it was reported that TAK-875 caused cholestatic liver injury by inhibiting hepatobiliary transporters, namely, BSEP, MRP2/3/4, NTCP, and OATP (Li et al. 2015, Otieno et al. 2017a, Wolenski et al. 2017). One of the direct effects of the bile acid inhibition by TAK-875 was the elevated serum total bile acids (TBAs) and total bilirubin both in rats and dogs (Wolenski et al. 2017). All these transporters are known to be polymorphic, and their genetic mutations could lead to the inter-individual differences in susceptibility to TAK-875-induced hepatotoxicity (Li et al. 2015).

6.5.3 Regulatory Factors of Liver Transporters

The roles of hepatic transporters in the generation of DILI are very complicated, and it is essential to characterize their regulatory factors in the liver. There are multiple levels of transporter regulation including transcriptional (activators and repressors), posttranscriptional (splice variants), chromosomal (epigeneticmodifications), translational (mRNA stability), and posttranslational (alteration of proteins) modifications (Klaassen and Aleksunes 2010b). But not all of these regulators have been comprehensively studied in respect of hepatic transporters and DILI. Here we picked out nuclear receptors (NRs), hepatocyte nuclear factors (HNF), nuclear factor (erythroid 2-related) factor 2 (Nrf-2), micro-RNA (miRNA), and pro-inflammatory factors as examples to highlight the potential mechanism of DILI caused by hepatic transporters alteration.

6.5.3.1 Nuclear Receptors (NRs)

Nuclear receptor (NR) target genes are involved in drug and bile acid transport and appear to be sensitive to drugs as well as bile acids.

It has been reported that farnesoid X receptor (FXR), pregnane X receptor (PXR), and the constitutive androstane receptor (CAR) are able to stimulate Mrp2 expression in rodents via shared binding sites (Kast et al. 2002). In particular, FXR can protect hepatic cells against bile acid overload by increasing the expression of BSEP and MRP2 (Synold et al. 2001, Xie et al. 2001) and decreasing the expression of transport proteins responsible for the uptake of bile acids into the hepatocyte (NTCP, OATP1B1) (Xie et al. 2001).

Besides, treatment of mice with PPARα agonists (clofibrate, fenofibrate, etc.) increases hepatic and intestinal Octn2 mRNA expression and carnitine levels in wild-type mice but not in PPARα-null mice, which will disturb the carnitine homeostasis (Ringseis et al. 2007, Maeda et al. 2008).

Considering the positive regulation of nuclear receptor on the hepatic transporters, administration of drugs targeting nuclear receptors may be a therapeutic strategy in transporter-related DILI. One case in point is that co-administration of nuclear receptor inducers may activate bile acid transporters (OATP/NTCP/BSEP/MRP2) and relieve the estrogen-induced cholestasis (Chen et al. 2013). However, the impacts on nuclear receptors may be very complicated. For example, it was reported that leflunomide, as an indirect PPAR α activator, may decrease Mrp2 while increasing Mrp3 expression and function (Wang et al. 2018).

6.5.3.2 Hepatocyte Nuclear Factors (HNF)

Hepatocyte nuclear factors (HNF) are the liver-specific regulators (Drewes et al. 1996). HNFs play important roles in both bile acid and drug metabolism by

regulating the expression of CYP enzymes. For example, HNF-1α controlled the expression of Cyp-2E1, which involved in the bio-activation of acetaminophen (APAP) to form NAPQI and, thus, APAP hepatotoxicity (Lee et al. 1996). HNF1α was reported to play an important role in the regulation of bile acid transport genes including Ntcp, Oatp1/2, Mrp, etc. (Arrese and Karpen 2002).

HNF-4α appears to be the most abundant transcription factor in the liver. It is the major trans-activator of bile acid-synthesizing genes, CYP7A1, CYP8B1, and CYP27A1, and major drug-metabolizing CYPs, CYP3A4, CYP2B6, CYP2C9, CYP2D6, demonstrated both in human and rodents. HNF-4α is also a key regulator of hepatic transporters. Downregulation of HNF-4α using siRNA suppressed Ntcp RNA expression up to 95% in mouseHepa1–6 cells (Geier et al. 2008). It also reduced MDR1, BSEP, MRP2, OATP1B1, and OCT1 mRNA level in human hepatocytes (Kamiyama et al. 2007).

These results implied the potential feasibility by activating HNF1α or HNF4α to relieve cholestasis and liver toxicity (by detoxification). In addition, it is also possible that some hepatotoxic drugs may exert their effects on hepatic transporters via an unknown HNF pathway.

6.5.3.3 Nuclear Factor (Erythroid 2-Related) Factor 2 (Nrf-2)

Nuclear factor (erythroid 2-related) factor 2 (Nrf-2) is the chief oxidative stress-responsive transcription factor that upregulates a wide spectrum of cytoprotective genes through its cognate enhancer antioxidant response element (Han et al. 2013).

Beyond its traditional role as an oxidative stress sensor, Nrf-2 is also a positive transcriptional regulator of human BSEP expression, and pharmacological activation of Nrf2 may be beneficial for cholestatic liver injury (Weerachayaphorn et al. 2009).

Similarly, Nrf2 helps to regulate the interaction between chemopreventive phytochemicals and detoxifying enzymes/transporters, which may help to reduce the cancer chemoprevention drug-induced toxicity (Wu et al. 2013).

The Nrf2 activator oltipraz induces Mrp3, Mrp4, Mdr1a, and Mdr1b mRNA in rat livers and MDR1, MRP2, MRP3, and BCRP in human hepatocytes (Cherrington et al. 2002, Maher et al. 2008).

6.5.3.4 Micro-RNA (miRNA)

More and more genetic polymorphisms have been identified, but only 10–30% of phenotype variations may be explained by genetic polymorphisms in drug transporter genes (Bonder et al. 2014). More evidence has indicated miRNAs as epigenetic modulators play an important role in the modulation of drug-metabolizing enzymes and transporters (DMETs) and nuclear receptors (NRs) (Hirota et al. 2017). Conversely, miRNA expression is clearly affected by the administration of common drugs, such as dexamethasone, vinblastine, fluoxetine, and enoxacin (He et al.

2015b) as well as the xenobiotic stressors, including chemical pollutants (Nakajima and Yokoi 2011, Yokoi and Nakajima 2013, Ning et al. 2014).

By RNA interference, miRNAs lead to inhibition of translational processes or messenger RNA (mRNA) degradation of their target genes encoding the hepatic transporters (Rukov et al. 2011, Haenisch et al. 2014), especially the efflux pumps of the ABC transporter family (Haenisch et al. 2014). When P-gp function was inhibited by mdr1-a siRNA, the increase of triptolide (TP) exposure in mice was quantitatively correlated to the enhanced hepatotoxicity (Kong et al. 2015). Simvastatin and atorvastatin were reported to induce miR-33 which represses both ABCB11 (BSEP) and ATP8B1, thus decreasing bile secretion and eventually leading to intrahepatic cholestasis (Allen et al. 2012).

During the past few years, several important works have been carried out to support the usage of miRNA as biomarkers for diagnosis and prognosis of DILI (Wang et al. 2009, Starkey Lewis et al. 2011). And the relationship between specific miRNA levels in the body fluids and the function of transporters need to be further explored.

6.5.3.5 Pro-inflammatory Cytokines

DILI-associated inflammation has been reported to cause pronounced changes in hepatic transporter expression. As we all know, acetaminophen (APAP)-induced liver injury is a typical case of DILI. Previous study found that MRP4 (Mrp4) was significantly upregulated in human (Barnes et al. 2007) or mouse (Aleksunes et al. 2006) liver following APAP treatment. Later the same laboratory suggested that Kupffer cell mediators (TNF-α, IL-1β) released in response to APAP were likely responsible for the induction of Mrp4 (Campion et al. 2008). Another systematic research indicated that IL-1β, TNF-α, and IL-6 could markedly impair expression of both sinusoidal and canalicular drug transporters in human hepatocytes (Le Vee et al. 2008, 2009).

These findings remind us that drugs that cause liver inflammation with pro-inflammatory cytokines released may also induce the alteration of hepatic transporters, thus resulting in the disturbance of bile circulation as well as the development of cholestasis and the alteration of drug pharmacokinetics (Moseley 1997, Slaviero et al. 2003).

6.5.3.6 Drugs Targeting Hepatic Transporters

Up till now, the only specific antidote for acute DILI remains N-acetylcysteine (NAC) for acetaminophen (APAP)-induced liver injury, and NAC proves to be beneficial in some cases of non-APAP DILI (Green et al. 2013, Singh et al. 2013). Researches on therapeutics dedicating for specific DILI have lagged behind (Lewis 2015). However, drugs that regulate the expression of transporters may have the protective effects against specific drug-induced liver injury (Baghdasaryan et al. 2014, Zhou et al.

2016, Trauner et al. 2017). For example, UDCA exerts anti-cholestatic action by stimulating Bsep and Mrp2 in rat liver, which may be extrapolated to human liver (Dombrowski et al. 2006). Some pharmacological ligands targeting FXR and TGR5 may exert their anti-cholestatic function by stimulating biliary BAs (BSEP) (Hegade et al. 2016), phospholipids (PLs) (MDR3) (Slijepcevic et al. 2015). Blockers or inducers of hepatobiliary transporters may be a new therapeutic target. For example, Myrcludex B, as a NTCP blocker, is reported to reduce hepatocellular bile acid uptake (Slijepcevic and Van De Graaf 2017). In human volunteers, biliary elimination of digoxin was increased on co-administration with rifampicin (a chronic P-gp inducer) resulting in decrease in AUC (Drescher et al. 2003).

6.5.4 Hepatic Drug Transporters and Drug-Induced Enteropathy and Diarrhea

Apart from the liver toxicity, hepatic transporters also play a role in the drug-induced gastrointestinal toxicity. The relevant transporters are often called hepatobiliary transporters, mainly including uptake transporters (NTCP, OATP) and export transporters (BSEP, MRP2, P-gp, BCRP). And the gastrointestinal toxicity of certain types of therapeutic agents often resulted from their concentrative excretion into the bile with the help of hepatobiliary transporters.

As has been verified that enterohepatic recirculation of a compound/drug occurs by biliary excretion and intestinal reabsorption, the bile circulation may notably affect pharmacokinetic parameters such as plasma half-life and AUC as well as bioavailability and toxicity of drugs (Malik et al. 2016).

Drug elimination into bile is mainly mediated by three ABC transporters: P-gp, BCRP, and MRP2. A large number of papers have reported that these transporters mediate the efflux of a large variety of drugs (Nies and Keppler 2007, Polgar et al. 2008, Cascorbi 2011).

However, the concentrative excretion into the bile of some therapeutic agents may be related to their gastrointestinal toxicity.

One such example is the gastrointestinal toxicity caused by antineoplastic agent, irinotecan. Early studies have demonstrated that active metabolite of irinotecan, SN-38, and its glucuronide (SN38-Glu) are mainly excreted into the bile via MRP2 both in rats and humans (Chu et al. 1997a, Chu et al. 1997b, Chu et al. 1998). SN-38 is metabolized to an inactive glucuronic acid conjugate (SN-38G) in the liver and hydrolyzed to SN-38 by β-glucuronidase in intestinal microflora (Di Paolo et al. 2006). The mechanism of irinotecan-induced diarrhea has not been fully clarified; however, SN-38 accumulated in intestinal tissues is believed to be responsible for the cytotoxicity (Kobayashi et al. 1999, Ikegami et al. 2002). The importance of biliary excretion in irinotecan-induced gastrointestinal toxicity was verified by a study where the co-administration of probenecid (Mrp2 inhibitor) reduced the late-onset diarrhea caused by irinotecan in rats (Horikawa et al. 2002).

Another example is the gastrointestinal toxicity of nonsteroidal anti-inflammatory drugs (NSAIDs). In about 70% of chronic NSAID users, significant small intestinal damage and even bleeding can be observed (Bjarnason et al. 1993, Graham et al. 2005). Recently, one case on indomethacin (INDO)-induced intestinal injury/bleeding was reported (Mayo et al. 2016). The results demonstrated that bile secretion played an important role in INDO-induced gut injury and appears to support enterococcal overgrowth of the intestine. It was thought that the inhibiting of cyclooxygenase (COX), the rate-limiting enzyme in synthesis of prostaglandins (PGs) by NSAIDs, is the major reason of for NSAID gastrointestinal toxicity (Schoen and Vender 1989). However, that couldn't explain why intestinal damage by NSAIDs occurs also in COX-1 knockout mice, demonstrating that topical (non-prostaglandin-mediated) mechanisms are involved (Darling et al. 2004). Besides, bile flow interruption in animal models completely prevents intestinal damage by parenterally administered NSAIDs (Djahanguiri et al. 1973), while the extent of intestinal injury had no correlation with the degree of inhibition of PG synthesis (Ligumsky et al. 1983, Ligumsky et al. 1990, Reuter et al. 1997).

The importance of bile salts in the mechanism of NSAID gastrointestinal toxicity has been recognized. Works from different groups support the concept that NSAIDs exert their detrimental action on the gastrointestinal mucosa because they reduce the protection of phosphatidylcholine against bile salt cytotoxicity (Barrios and Lichtenberger 2000). Strategies aiming to increase biliary phospholipid concentration (such as FXR modulators) may help to reduce gastrointestinal toxicity during NSAID therapy (Fiorucci et al. 2011).

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Chapter 7
Roles of Renal Drug Transporter in Drug Disposition and Renal Toxicity

Xinning Yang and Li Han

Abstract The kidney plays an important role in maintaining total body homeostasis and eliminating toxic xenobiotics and metabolites. Numerous drugs and their metabolites are ultimately eliminated in the urine. The reabsorption and secretion functions of the nephron are mediated by a variety of transporters located in the basolateral and luminal membranes of the tubular cells. In the past decade, many studies indicated that transporters play important roles in drug pharmacokinetics and demonstrated the impact of renal transporters on the disposition of drugs, drug-drug interactions, and nephrotoxicities. Here, we focus on several important renal transporters and their roles in drug elimination and disposition, drug-induced nephrotoxicities and potential clinical solutions.

Keywords SLC transporters · ABC transporters · Drug disposition · Nephrotoxicity

7.1 Introduction

This chapter primarily focuses on seven renal transporters recommended by regulatory agencies for routine assessment of inhibition potential of investigational drugs, i.e., organic anion transporters (OAT1 and OAT3), organic cation transporter 2 (OCT2), multidrug and toxin extrusions (MATE1 and MATE2/K), P-glycoprotein (P-gp), and breast cancer resistance protein (BCRP). These transporters work in conjunction to accomplish active excretion of structurally diverse compounds from the blood into urine. Several other renal transporters also known to be involved in disposition, efficacy, or toxicity of drugs or endogenous compounds are not covered

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here but discussed in depth by literature reviews. For example, monocarboxylate transporters (MCTs) are responsible for reabsorption of γ-hydroxybutyric acid (GHB), urate transporter 1 (URAT1) is involved in excretion of uric acid and a target for drugs to treat gout, and OAT2 was recently found as a major transporter mediating renal active secretion of creatinine (Morris and Felmlee 2008; Deeks 2017; Shen et al. 2017). In addition, the scope of this chapter is limited to drugs or ingredients in herbal medications and their interactions with transporters and related systemic or renal toxicities. The roles of aforementioned transporters in the disposition and nephrotoxicity of environmental contaminants, such as mercury and paraquat, are not discussed in this chapter but covered in details by other reviews (Wang and Sweet 2013; George et al. 2017).

7.2 Roles of Renal Transporters in Drug Disposition in the Kidney

7.2.1 OAT1 and OAT3

In the past decade, the important roles of OAT1 and OAT3 in drug disposition have been recognized with a number of drugs undergoing substantial renal elimination including diuretics, antihypertensive, antibiotics, antivirals, and anticancer agents (Wang and Sweet 2013). Although most of the substrates of OAT1/OAT3 are anions or zwitterions, some organic cation drugs can also be transported by these transporters. For example, sitagliptin is a substrate of OAT3 (but not OAT1) and cimetidine transported by OAT1 and preferably by OAT3 (Tahara et al. 2005; Chu et al. 2007). Several OAT1/OAT3 substrates suitable for clinical drug-drug interaction (DDI) studies to detect inhibitory effect of a drug on OAT1/OAT3 in humans are listed here: (1) adefovir and ganciclovir considered more specific for OAT1; (2) bumetanide, cefaclor, methotrexate, oseltamivir carboxylate, and penicillin G regarded as OAT3-specific substrates; (3) while ceftizoxime, ciprofloxacin, and furosemide considered as dual substrates of OAT1 and OAT3 (Lee et al. 2017). These drugs were found to be transported by OAT1/OAT3 in more than one in vitro study but not significantly by organic cation transporters (thus cimetidine was excluded as it was also a substrate of OCT2) (Tahara et al. 2005). The substantial contribution of OAT1/OAT3 to the elimination of these drugs in humans was confirmed by clinical DDI studies where co-administration with probenecid, a well-known potent inhibitor of OAT1 and OAT3, increased the *AUC* (area under the curve of plasma concentration-time profile) of these drugs by at least 50% (Aherne et al. 1978; Welling et al. 1979; Smith et al. 1980; LeBel et al. 1983; Odlind et al. 1983; Jaehde et al. 1995; Vree et al. 1995; Cimoch et al. 1998; Hill et al. 2002; Landersdorfer et al. 2010; Maeda et al. 2014). It should be noted that the above list is not a comprehensive one and may be supplemented with other drugs when more OAT1/OAT3 substrates are revealed.

Significant increases of drug concentrations in circulation due to inhibition of OAT1/OAT3 may potentially lead to more adverse events associated with systemic exposure (Ivanyuk et al. 2017). On the other hand, inhibition of OAT1/OAT3 may limit the entrance of drugs with nephrotoxicity into proximal tubular cells and thus provides protectant effect. A well-known example is cidofovir, for which nephrotoxicity is the major dose-limiting toxicity (VISTIDE® USPI). Following 3 mg/kg or 5 mg/kg intravenous infusion doses of cidofovir in patients, its *AUC* was increased by 29% or 46%, respectively, with the presence of probenecid compared to administration of cidofovir alone, while renal clearance (CL_r) of cidofovir was decreased by 36%. It was suggested that, at 5 mg/kg dose of cidofovir, probenecid blocked tubular secretion of cidofovir and reduced its renal clearance to a level close to glomerular filtration (Cundy et al. 1995). Cidofovir was identified as an OAT substrate by multiple studies and found to be more selective to OAT1 than OAT3. The uptake of cidofovir in HEK293 cells (human embryonic kidney) stably expressing OAT1 or OAT3 was 22.5 ± 0.4 and 0.295 ± 0.024 μL/mg protein/5 min, respectively, in comparison to 0.135 ± 0.010 μL/mg protein/5 min in control cells (Uwai et al. 2007). In CHO cells transfected with OAT1, the intracellular level of cidofovir was 186-fold of that in CHO control cells, and accordingly cidofovir showed 400-fold higher cytotoxicity in CHO-OAT1 cells compared to CHO cells (Ho et al. 2000). Probenecid significantly reduced OAT1-mediated uptake of cidofovir and resultant cytotoxicity effect. Thus, the approved US drug product labeling of cidofovir (VISTIDE®) requires it be administered with probenecid, to reduce renal tubular uptake of cidofovir and consequent renal toxicity.

Another example where uptake of drug into tubular cells is modified to improve its renal toxicity profile is tenofovir, a nucleotide analog HIV-1 reverse transcriptase inhibitor and a hepatitis B virus reverse transcriptase inhibitor. Tenofovir has poor membrane permeability and thus is not suitable for oral administration due to low bioavailability (Ray et al. 2016). It was initially developed as a prodrug, tenofovir disoproxil fumarate (TDF), which was approved in the USA as a stand-alone product (VIREAD®) and also as an active ingredient of combinational products (e.g., elvitegravir, cobicistat, emtricitabine, plus TDF, marketed as STRIBILD®). After administered as TDF, tenofovir is primarily eliminated through urine as unchanged drug and is associated with renal adverse events including acute renal failure (AKI) and Fanconi syndrome (renal tubular injury with severe hypophosphatemia) (VIREAD® USPI). Recently, another prodrug of tenofovir, tenofoviral afenamide fumarate (TAF), was developed and approved as a component of several combinational products (e.g., elvitegravir, emtricitabine, cobicistat, plus TAF, marketed as GENVOYA®) and also as a stand-alone product (VEMLIDY®). The rationale for development of TAF was lowering tenofovir concentrations and improving renal safety (Ray et al. 2016). This was achieved through modifying the pharmacokinetic properties of this prodrug and thus changing the distribution of tenofovir into pharmacological target cells and off-target sites including the kidney.

The efficacy of tenofovir actually comes from its active metabolite, tenofovir diphosphate, which is formed by cellular enzymes in target cells. On the other hand, nephrotoxicity of tenofovir is associated with its accumulation in kidney tubular cells.

Tenofovir is a substrate of OAT1/OAT3 and multidrug resistance protein4 (MRP4), which are responsible for uptake of tenofovir from blood into tubular cells (primarily by OAT1) and excretion out of cells into urine, respectively (Ray et al. 2006; Uwai et al. 2007). Overexpression of OAT1 increased cytotoxicity of tenofovir in HEK293 cells, while co-transfection with MRP4 reduced cell sensitivity to tenofovir's toxicity (Stray et al. 2013). While TAF is also eventually converted to tenofovir, there is notable difference from TDF in that TAF is more stable. In humans, TAF had a half-life ($t_{1/2}$) around 0.5 hr, while TDF was much more rapidly converted to tenofovir (Kearney et al. 2004). Moreover, TAF is more lipophilic than tenofovir and is permeant into cells by passive diffusion (and also by organic anion transporter polypeptide (OATP) 1B1 and 1B3 present in hepatocyte cells). After penetrating into cells, TAF undergoes hydrolysis and subsequent transformation to tenofovir which is further converted to tenofovir diphosphate (Ray et al. 2016). Importantly, TAF is not a substrate of OAT1/OAT3 and does not exhibit OAT-dependent cytotoxicity (Bam et al. 2014). Therefore, the relatively sustained presence of TAF in systemic circulation compared to TDF leads to more accumulation of tenofovir in pharmacology target cells, while presumably less distribution into tubular cells due to absence of OAT-mediated uptake.

The favorable shift of tenofovir distribution into target cells by TAF versus TDF was proved in vivo first in dogs where peripheral blood mononuclear cells (PBMC) to plasma ratios of tenofovir were 4.7 and > 160, respectively, with administration of equivalent oral doses of TDF and TAF (Lee et al. 2005). This was further demonstrated in humans. In HIV-infected subjects, tenofovir (TFV) *AUC* was reduced by ~90% in subjects administered with elvitegravir/emtricitabine/cobicistat/TAF (TAF 10 mg equivalent to 6 mg of TFV) compared to elvitegravir/emtricitabine/cobicistat/TDF (TDF 300 mg equivalent to 136 mg of TFV), while the intracellular *AUC* in PBMCs of the active metabolite tenofovir diphosphate was similar (NDA 207561 Clinical Pharmacology review). In a monotherapy study in which HIV-infected subjects were administered TDF 300 mg, TAF 8 mg, TAF 25 mg, and TAF 40 mg for 10 days, anti-HIV activity (log reduction in HIV RNA) was similar for TAF 8 mg relative to TDF and increased for the TAF 25 mg and 40 mg groups. While maintaining efficacy, lower dose of TAF and resulting lower tenofovir exposure in systemic circulation and the kidney appeared to have a reduced impact on renal function (Aloy et al. 2016). Recently, there were two case reports that tenofovir-induced Fanconi syndrome was resolved after patients with HIV and/or HBV were switched from TDF to TAF treatment (Karris 2017; Mothobi et al. 2018). However, it should be aware that a case of occurrence of acute kidney injury was also reported in a patient with HIV/HCV coinfection (Serota et al. 2018). Overall, TAF seems promising to provide better renal tolerance, while follow-up evaluation of long-term renal safety is warranted to confirm that TAF conveys better renal safety profile than TDF in the long run.

7.2.2 OCT2, MATE1, and MATE2/K

Organic cation transporters play a critical role in renal excretion of many endogenous organic cations, cationic drugs, and xenobiotics. Among a number of transporters present in tubular proximal cells, OCT2 is one of the most extensively studied, which is a basolateral transporter and responsible for uptake of drugs from systemic circulation into kidney cells. MATEs (MATE1 and MATE2/K) were discovered more recently as efflux transporters present in lumen membrane of tubular cells which pump drugs out of kidney cells into urine and sometimes couple with OCT2 to mediate renal active secretion of drugs. OCT2 and/or MATEs are involved in the disposition and toxicity of drugs, DDIs, and drug-endogenous substrate interactions. The roles of OCT2 and/or MATEs in nephrotoxicity of cisplatin are described in later part of this chapter. Herein, a few examples are presented to illustrate the impact of OCT2 and/or MATEs on drugs' renal elimination, interaction with other drugs, and some unresolved issues.

Memantine is an NMDA receptor antagonist commonly prescribed for patients with moderate to severe Alzheimer's disease and undergoes substantial renal elimination which involves active secretion. According to the US drug product labeling, the clearance of memantine was reduced by about 80% under alkaline urine conditions at pH 8 (NAMENDA® USPI). Hence, alkalization of urine may lead to accumulation of the drug and possible increase in adverse effects. Urine pH is altered by diet, drugs (e.g., carbonic anhydrase inhibitors, sodium bicarbonate), and clinical state of the patient (e.g., renal tubular acidosis or severe infections of the urinary tract). Therefore, memantine should be used with caution under these conditions. Based on these findings, it was speculated that memantine may be a substrate of MATE1 and/or MATE2/K, since MATEs-mediated transport is driven by proton gradient and lower pH in urine relative to kidney cells will stimulate efflux of MATEs. Actually, some drugs with similar characteristics (i.e., cationic compounds excreted substantially through urine and is sensitivity to urine pH change) have been demonstrated as substrates of OCT2 and MATEs, such as methamphetamine and amphetamine, two drugs used to treat attention-deficit hyperactivity disorder (ADHD). According to the labeling of DESOXYN® and DEXEDRINE®, co-administration of methamphetamine or amphetamine (active metabolite of methamphetamine, also approved as a drug by itself) with urinary alkalinizing agents (e.g., acetazolamide, some thiazides) may increase blood concentration of methamphetamine and/or amphetamine and thus potentiate their actions. On the other hand, urine-acidifying agents (e.g., ammonium chloride, sodium acid phosphate, methenamine salts) may decrease blood levels of these drugs and hence impair their efficacy. It was later found out that both drugs are substrates of OCT2, MATE1, and MATE2/K, but not OCT1 and OCT3, in HEK293 cells transfected with individual transporters compared to control cells (Wagner et al. 2017).

The hypothesis that memantine is also transported by MATEs is not proved until 2017 when a publication reported that memantine was a substrate of MATE1

(Müller et al. 2017). Initially, uptake experiments in HEK293 cells transfected with OCT2, MATE1, or MATE2/K did not show memantine significantly transported by any of these transporters. However, further investigation using MDCK cell monolayers transfected with MATE1 (apical) or co-transfected with OCT2 (basolateral, corresponding to renal blood side) and MATE1 (apical, corresponding to renal lumen side) demonstrated vectorial, basal to apical transport of memantine (added into basal compartment) which was inhibited by cimetidine, a known OCT2 and MATEs inhibitor. Consistently, the cellular accumulation of memantine in MDCK cells transfected with MATE1 or OCT2 and MATE1 was lower than control cells, and such difference was diminished in the presence of cimetidine. In addition, the authors also evaluated the effect of apical compartment pH change (from 5.0 to 8.0, while basal compartment pH was kept at 7.3) in MDCK cells co-transfected with OCT2 and MATE1 compared to mock cells. It was found that transcellular transport of memantine from basal to apical decreased, while its cellular accumulation increased (memantine was added in basal compartment) when apical chamber pH increased, which was consistent with in vivo observation that alkalization of urine reduced memantine clearance. Collectively, these results suggested that memantine was a substrate of MATE1. This may explain a clinical finding that a patient with impaired renal function suffered from severe myoclonus and delirium after trimethoprim was added onto her existing therapy with memantine and the symptom was resolved after discontinuation of trimethoprim (Moellentin et al. 2008). Trimethoprim was reported by multiple studies as an inhibitor for OCT2, MATE1, and MATE2/K, with IC_{50} values in the order of OCT2 > MATE1 > MATE2/K (Müller et al. 2013; Lepist et al. 2014; Chu et al. 2016; Mathialagan et al. 2017). At its clinical relevant concentrations, trimethoprim is expected to inhibit these transporters in humans (Zhong et al. 2016). In fact, trimethoprim was shown to increase the exposure to metformin, a probe substrate for OCT2 and MATEs, by 30%–37% in healthy subjects (Müller et al. 2015). It will be interesting to know if memantine is also transported by MATE2/K which the authors have not evaluated. In addition, although there were discordant findings between HEK293 uptake and MDCK transcellular experiments, neither of them found memantine as an OCT2 substrate. Yet, an earlier study showed that memantine uptake was greater in OCT2 mRNA-injected oocytes than in water-injected oocytes (Busch et al. 1998). Thus, it is warranted to further investigate whether memantine is an OCT2 substrate, and if it is confirmed that memantine is not a substrate of OCT2, which other cationic renal transporter(s) may be responsible for memantine uptake into tubular cells.

The memantine example indicates the importance of using different cell systems for OCT2/MATEs transport assessment to have a better mechanistic understanding and also highlights the complexity of molecular mechanisms for renal active secretion and thus the need to acquire sufficient in vitro evidence than simply make an assumption based on results from clinical studies. This is further illustrated by dofetilide, a drug used for the maintenance of normal sinus rhythm in patients with atrial fibrillation/atrial flutter (TIKOSYN® USPI). Among a number of substrates of OCT2 and/or MATEs, metformin and dofetilide are two drugs that often raise clinical concerns, since metformin is the first-line therapy for type 2 diabetes

affecting a large number of patients and has a rarely occurring but severe adverse event (lactic acidosis) (GLUCOPHAGE® USPI), while dofetilide is a narrow therapeutic index drug due to its QT prolongation effect and can cause life-threatening ventricular arrhythmias. Therefore, concomitant use of these drugs with OCT2 and/or MATE inhibitors may have clinically significant consequences (increasing adverse events). Although there is extensive evidence suggesting that metformin is a substrate of OCT2 and also MATEs, there is scarce published evidence about whether dofetilide is actually transported by OCT2 and/or MATEs (Masuda et al. 2006; Meyer zu Schwabedissen et al. 2010; Reese et al. 2013). Hence, it appears that dofetilide is commonly presumed to be a substrate of OCT2 and MATEs based on the following in vivo observations: (1) dofetilide, a cationic drug, is predominantly eliminated through urine as unchanged drug with significant renal active secretion; (2) several drugs (e.g., cimetidine, trimethoprim) that inhibit OCT2 and/or MATE transporters are known to increase dofetilide plasma concentrations in humans (Abel et al. 2000). Despite lack of direct in vitro evidence, some drug developers proposed to contraindicate concomitant use of dofetilide with their drugs (e.g., dolutegravir, bictegravir) that are inhibitors of OCT2 and/or MATEs (Gillette et al. 2014).

In vitro assessment regarding whether dofetilide is a substrate of renal transporters was not available until 2018 (Uddin et al. 2018). In contrast to common perceptions, dofetilide was found not transported by OCT2. In HEK293 cells transfected with OCT2 or MATE1, the uptake of model substrates tetraethylammonium (TEA) and metformin was >29-fold and > 34-fold, respectively, of that in vector-transfected cells. While dofetilide transport was increased by five-fold in MATE1 transfected cells that was time-dependent and saturable, there was no apparent difference between HEK293 cells expressing OCT2 and mock cells. This surprising finding casts doubt on the validity of proposal or recommendation to prohibit concomitant use of dofetilide with drugs that are relatively specific inhibitors of OCT2. For example, dolutegravir was found inhibiting OCT2 more potent (at least >40-fold) than MATE1 and MATE2/K in a study where IC_{50} of dolutegravir was determined as 0.066 μM for OCT2, 4.67 μM for MATE1, and > 100 μM for MATE2/K using TEA as substrate (Lepist et al. 2014). This trend was later confirmed by another lab using metformin as substrate which reported IC_{50} values of 0.21 μM, 3.6 μM, and 12.5 μM of dolutegravir for OCT2, MATE1, and MATE2/K, respectively (Chu et al. 2016). However, the relative specificity of dolutegravir toward OCT2 than MATE1 was not demonstrated in another study using creatinine as substrate, where IC_{50} of dolutegravir for OCT2 was measured as 8.25 μM similar to 5.8 μM for MATE1 (Mathialagan et al. 2017). This discrepancy might be attributed to substrate-dependent inhibitory potency. In the latter study, IC_{50} of dolutegravir for MATE2/K was also determined using metformin as substrate, and the value obtained (9.3 μM) was several folds lower than that with creatinine (49.3 μM) but closer to the former report (12.5 μM).

Nevertheless, these findings underscore the need for further evaluations (1) to investigate whether dofetilide is a substrate of OCT2 and, if not, which other basolateral carrier(s) may be involved; (2) to confirm MATE1-medated dofetilide transport; (3) to test whether dofetilide is transported by MATE2/K; and (4) to

determine the IC_{50} of several drugs that might be more potent inhibitors against OCT2 than MATEs (e.g., dolutegravir, bictegravir) toward MATE1 and/or MATE2/K using dofetilide as substrate and compared to in vivo concentrations (e.g., unbound or total C_{max}) of these drugs to see whether there is potential for them to inhibit MATE-mediated dofetilide transport in vivo. Such assessments will resolve the following questions: (1) is it appropriate or necessary to prohibit patients using dofetilide in clinical trials for an investigational drug that is an inhibitor only for OCT2; (2) is it scientifically justified to recommend in drug labelings avoiding or even contraindicating concomitant use of dofetilide with specific inhibitors of OCT2. Same questions are also applied to MATE1 and/or MATE2/K inhibitors. The answers will have direct impact on drug development (e.g., exclusion criteria and prohibited medication list in clinical trials) and clinical use of polydrugs by patients.

7.2.3 P-gp

P-gp is one of the efflux transporters present in luminal membrane of proximal tubular cells which is responsible for exporting compounds from kidney cells into urine. P-gp has a wide substrate spectrum, among which digoxin is the most often studied substrate in clinical DDI studies with P-gp inhibitors due to (1) its minimal metabolism in humans (thus CYP3A-mediated interaction can be excluded as a confounding factor when interpreting results (some P-gp inhibitors are also CYP3A inhibitors)) and (2) it being a narrow therapeutic index drug (therefore a limited extent of change in digoxin exposure may still be clinically meaningful) (Chu et al. 2018). Quinidine, a well-recognized P-gp inhibitor, reduced systemic clearance of digoxin (given intravenously) by 34% to 64% (median: by 43% across studies) and thus increased digoxin *AUC* to 1.5–2.8 folds (median: 1.8-fold) of that with the absence of quinidine, as reported by a number of dedicated DDI studies conducted in healthy subjects or patients (Hager et al. 1981; Ochs et al. 1981; Schenck-Gustafsson and Dahlqvist 1981; Fenster et al. 1982; Pedersen et al. 1983b; Fenster et al. 1984). *CLr* of digoxin was also decreased by 28%–61% (median: by 35% reduction), indicating that quinidine may inhibit P-gp in proximal tubular cells. Comparable increase of digoxin AUC was observed in studies where digoxin was given orally, i.e., 1.8-fold and 2.7-fold in groups of patients and healthy volunteers, respectively, in the presence of quinidine compared to digoxin given alone, with CL_r of digoxin decreased by 33% or 48% (Pedersen et al. 1983c; Rameis 1985). However, alteration of CL_r of digoxin was not always aligned with the change of its total clearance. Two studies were conducted in healthy subjects who received intravenous single doses of 1 mg digoxin in the presence or absence of similar dosing regimen (80 mg, three times a day) of verapamil, another well-known P-gp inhibitor, with one study reporting a 35% reduction in systemic clearance of digoxin and 21% decrease in its CL_r, while the other study showing only 18% decline of total clearance and a small increase (5%) for CL_r (Pedersen et al. 1981; Rodin et al. 1988). The reason for

such discrepancy remains unknown, since the PK sampling periods were similar (72 h vs. 78 h) and in both studies the samples were analyzed using commercial radioimmunoassay kits. Inconsistent findings for digoxin CL_r changes with co-administration of verapamil were also observed when digoxin was given orally as multiple doses in healthy volunteers or patients. Although the *AUC* or plateau plasma concentrations (C_{avg}) of digoxin in general showed consistent trend (29%–77% increase, with an average around 60%, in the presence of verapamil versus absence), the changes of digoxin CL_r varied from a 53% reduction to a 13% increase (Klein et al. 1982; Pedersen et al. 1982; Belz et al. 1983; Rodin et al. 1988; Hedman et al. 1991). In comparison to quinidine, it seems that verapamil may have less effect on proximal tubular P-gp, since quinidine consistently reduced digoxin CL_r across studies. This might partially explain the overall smaller DDI effect of verapamil on pharmacokinetics of intravenously administered digoxin compared to quinidine (by 23%–59% increase in digoxin *AUC* with verapamil vs. by 51%–178% with quinidine) (Pedersen et al. 1983a). These findings also suggested the utility of collecting urine samples and characterizing renal elimination parameters (e.g., *CLr* and Ae, amount of drug excreted into urine in unchanged form) in terms of better understanding the mechanism(s) for observed interactions.

Increased concentrations of digoxin due to P-gp inhibition were shown leading to more adverse events. For example, in a study conducted in 36 healthy males who were given digoxin orally to steady state (0.125 mg, thrice daily) and received placebo or P-gp inhibitors, a 69% or 118% increase in digoxin plasma concentrations was observed in the groups receiving three times daily oral doses of verapamil (80 mg or 120 mg) or quinidine (250 mg) compared to the periods when those subjects received digoxin alone, and accordingly digoxin CL_r was decreased by 32% and 42%, respectively. The increase of digoxin concentration resulted in more glycoside effects, as measured by the shortening of systolic time intervals and flattening of T wave. There was a linear correlation between digoxin concentration and changes in mean corrected electromechanical systole and T wave flattening (Belz et al. 1983). In another study performed in 49 patients receiving once-daily dose of 0.25 mg digoxin orally for 2 weeks and then along with verapamil 240 mg/day in three divided doses, serum digoxin levels were increased from 0.76 ± 0.54 ng/mL (mean ± SD) to 1.31 ± 0.54 ng/mL with the presence of verapamil. Digoxin CL_r was only measured in seven patients. Except one patient who had CL_r slightly increased (and digoxin serum concentration was not influenced by verapamil in this patient), all the others had CL_r decreased (from 55.1 ± 12.3 mL/min to 26.1 ± 9.7 mL/min). Signs or symptoms of digoxin toxicity (ventricular premature complexes in ECG) were observed in seven patients during combined digoxin and verapamil treatment, with five out of these seven patients having digoxin concentration fall in the toxic range (greater than 2 ng/mL). The rhythm disturbances and symptom were resolved when the dose of digoxin or verapamil was decreased (Klein et al. 1982). Due to concerns with P-gp inhibition-mediated DDI and resulting increase of adverse events, the labeling of LANOXIN recommends measure serum digoxin concentrations before initiating a number of concomitant drugs that are P-gp inhibitors, e.g., amiodarone, clarithromycin, dronedarone,

erythromycin, itraconazole, lapatinib, propafenone, quinidine, ranolazine, ritonavir, telaprevir, and verapamil. Digoxin dose may need to be reduced by 30–50% (or adjusting dosing frequency), and continued monitoring is recommended (LANOXIN USPI).

7.2.4 BCRP

BCRP, an efflux transporter encoded by *ABCG2* gene, is localized at brush-border membranes in the proximal tubules in the kidney and mediates exporting compounds from kidney cells into urine. BCRP transports a wide array of substrates, including statins, steroids, folate, anticancer drugs, sulfated conjugates (Merino et al. 2005; Robey et al. 2005; Ando et al. 2007; Pan et al. 2007), some other xenobiotics (Suzuki et al. 2003), as well as uric acid (Woodward et al. 2009).

SC-62807, a major metabolite of celecoxib produced by CYP2C9 (Tang et al. 2000), is a substrate of BCRP. A recent study utilized [^{11}C]-SC-62807 to clarify the renal elimination mediated by BCRP by positron emission tomography (PET) imaging. The study demonstrated that the renal excretion of [^{11}C]-SC-62807 was significantly reduced in *Abcg2*$^{-/-}$mice (99% ± 1% lower than wild animals) and the systemic exposure of [^{11}C]-SC-62807 was increased. The marked decrease of its CL_r in *Abcg2*$^{-/-}$mice clearly indicates the contribution of BCRP to efflux of [^{11}C]-SC-62807 into the urine (Takashima et al. 2013).

It is evident that a substantial fraction of BCRP substrates are chemotherapeutics and also that a considerable substrate specificity overlap exists between BCRP and other members of the ABC superfamily such as P-gp and multidrug resistance-associated protein 2 (MRP2) (Caetano-Pinto et al. 2017). So the function of BCRP may be covered up by other transporters. For example, methotrexate (MTX), an antifolate drug, is frequently used in the treatment of acute leukemia and rheumatoid arthritis. Research showed that MTX is a substrate of BCRP (Volk et al. 2002; Volk and Schneider 2003). MTX is primarily excreted in urine in its unchanged form (Bleyer 1977); Henderson et al. showed that in mice at a dose of 15 mg/kg, 60–80% of intravenous MTX dose was excreted into the urine (Henderson et al. 1965). At a dose of 100 mg/kg, 28% of the intravenously administered dose of MTX was excreted in urine in wild-type mice, while in *Abcg2*$^{-/-}$ mice or mice given BCRP inhibitor pantoprazole, the urinary excretion was not significantly different from wild mice (Pauline et al. 2004). This work suggests that absence of BCRP or inhibition of BCRP by pantoprazole had no significant influence on renal clearance of MTX, and the increase of MTX exposure was mainly driven by reduced hepato-biliary elimination.

A recent study implicated that resveratrol (RES), a substrate of BCRP, could prevent renal injury caused by MTX via upregulation of renal BCRP (El-Sheikh et al. 2016). Co-administration of RES and MTX in rats caused significant increase in renal expression of BCRP (over seven times), and the renal nitrosative stress and the apoptosis of MTX-induced renal injury were reduced. However, whether the CL_r

and concentration of MTX were changed with the presence of RES were not evaluated in that study.

Species differences in BCRP expression levels have been reported; the expression of BCRP in human kidney is 50-fold lower than in rats (Huls et al. 2008; Fallon et al. 2016). Considering the much lower expression of BCRP in human kidney than rodents, the renal elimination mediated by BCRP might have less influence in human. Nevertheless, BCRP has been shown to play a role in homeostasis of urates and in patient response to allopurinol, a first-line treatment of hyperuricemia which causes gout (Cleophas et al. 2017). Genome-wide association studies (GWAS) revealed that carriers of BCRP gene alleles (rs2231142) that encodes Q141K variant had worse response to allopurinol (Wen et al. 2015; Brackman et al. 2019). The underlying mechanisms remain unclear. It was speculated that the observations may be due to 1) less intestine and/or renal excretion of uric acid that is a substrate of BCRP and resulting higher serum uric acid level; 2) lower concentration of allopurinol in tubule fluid because of less BCRP-mediated renal excretion of allopurinol, and in turn less inhibitory effect of allopurinol on URAT1, a transporter responsible for reabsorption of uric acid.

7.3 Contribution of Renal Drug Transporters to Drug-Induced Toxicity

The kidney is a major target for drug-induced toxicity. Renal drug transporters may account for some drug-induced nephrotoxicity because of their roles in the accumulation of drug in the kidney. Here we focus on the major renal drug transporters (OAT and OCT) and their involvement in renal toxicity.

7.3.1 Aristolochic Acid-Induced Nephropathy

Aristolochic acid (AA) is the main component that causes herbal nephropathy (CHN), Balkan endemic nephropathy (BEN), and renal cell carcinoma (RCC) (Tatu et al. 1998; Arlt et al. 2002). AA is composed of many nitrophenanthene carbocylic acids. Aristolochic acid I (AAI) and aristolochic acid II (AAII) are the two major components (Kumar et al. 2003) of AA, while AAI is the major toxic component causing aristolochic acid nephropathy (AAN) (Ding et al. 2005; Shibutani et al. 2007). Since the identification of AA with nephrotoxicity in the 1990s, the pathogenesis of AA kidney damage has been extensively studied.

After being taken into human body, AAI produces aristololactam nitrogen ions under the catalysis of nitroreductase, which further interact with DNA to form adducts. The AAI-DNA adduct can bypass molecular stagnation and repair, leading to the specific AT to TA transversion in p53 gene, which finally causes kidney

failure and urinary tract cancer (Jadot et al. 2017). Some other studies also showed that AAI may directly cause acute renal toxicity and apoptosis through the mitochondrial cell death pathway in renal tubular cells (Qi et al. 2007).

As typical organic anion transporters, OAT1 and OAT3 are responsible for the transportation of AAI into renal tubular cells and the subsequent nephrotoxicity. hOAT1 and hOAT3 exhibit high affinity with AAs (Babu et al. 2010). Xue et al. reported that AAs are substrates of OAT1 and OAT3. The concentration of AAI in hOAT1- and hOAT3-HEK293 cells was higher than that in mock cells (Xue et al. 2011), and the AAI-DNA adducts were significantly higher in cells expressing hOAT1 (1.8-fold) and hOAT3 (1.2-fold) (Bakhiya et al. 2009). The renal concentrations of AAI in OAT1 and OAT3 knockout mice were significantly reduced compared with wild-type animals, so did the kidney lesions (Xue et al. 2011).

The AAN can be reduced by probenecid, an OAT1 and OAT3 inhibitor. Probenecid inhibited AA entry into human OAT-transfected HEK293, reduced AA-DNA adduct formation, and preserved cellular viability (Bakhiya et al. 2009). Probenecid markedly reduced the renal concentration of AAI in mice, which indicates that OAT inhibition decreased tubular uptake and renal accumulation of AAI and its metabolites. The urinary excretions of AAI were also greatly reduced by probenecid. In contrast, the biliary excretions of AAI were substantially increased (Xue et al. 2011). The results suggested OAT inhibition prevented AAI-induced acute kidney injury and significantly reduced tubular necrosis, apoptosis, and renal fibrosis (Baudoux et al. 2012).

7.3.2 Methotrexate-Induced Nephrolithiasis

MTX, a folate antagonist, is used for the treatment of rheumatoid arthritis and cancer (Claudino et al. 2016). MTX can be safely administered at a low dosage, while the high-dose methotrexate (e.g., over 1000 mg/m^2 (Frei et al. 1980, Ackland and Schilsky 1987)) may cause serious adverse reactions, such as intestinal injury, nephrolithiasis, hepatotoxicity, and suppression of bone marrow. MTX is primarily excreted in urine via glomerular filtration and active tubular secretion (Bleyer 1977). Renal failure caused by the high-dose methotrexate may be due to overexcreted MTX in the renal tubule leading to crystal nephropathy (Green et al. 2006), and renal dysfunction may lead to a marked enhancement of MTX toxicities (Frei et al. 1980; Stark et al. 1989).

Many studies showed that human OAT1 and OAT3 mediated the renal uptake of MTX. The concentration of methotrexate increased in hOAT1 and hOAT3-transfected proximal tubule cells (Takeda et al. 2002). It has also been reported that co-administration of OAT inhibitors decreased CL_r of MTX. For example, MTX accumulation was markedly inhibited in rat kidney slices and hOAT1-HEK293 and hOAT3-HEK293 cells after giving an OAT inhibitor rhein. The systemic exposure of MTX in rats was strongly increased, while the damage of MTX was attenuated when simultaneously given with rhein (Liu et al. 2017). Other OAT inhibitors,

non-steroid anti-inflammatory drugs (NSAID) (Maeda et al. 2008) and resveratrol (Jia et al. 2016), also reduced MTX-induced nephrotoxicity. Thus, inhibiting OATs may be a potential strategy in reducing MTX nephrotoxicity.

7.3.3 Cisplatin-Induced Nephrotoxicity

Cisplatin is one of the most widely used chemotherapy drugs, because of its broad spectrum against cancers. However, its nephrotoxicity limits its usage in clinic. Cisplatin nephrotoxicity can present in a number of ways. Acute kidney injury occurs in 20–30% of patients taking cisplatin (Hartmann et al. 1999).

Cisplatin is cleared through glomerular filtration and tubular secretion in the kidney (Yao et al. 2007). The transporters involved in the cellular transport of cisplatin include the copper transporter-1 (Ctr1) (Ishida et al. 2002), the copper transporter-2 (Ctr2) (Blair et al. 2009), OCT2 (Ciarimboli et al. 2005; 2010), and MATE1 (Yonezawa et al. 2006; Nakamura et al. 2010). While Ctr1 and Ctr2 are ubiquitously expressed, OCT2 and MATE1 are highly expressed in kidneys.

Many studies indicated that OCT2 played a dominant role in cisplatin uptake. Ciarimboli et al. showed that the accumulation of cisplatin in human OCT2-transfected HEK293 cells was higher than in wild-type cells and proved that the OCT2 inhibitor cimetidine effectively suppressed cisplatin cellular toxicity (Ciarimboli et al. 2005). With cimetidine, the accumulation of cisplatin in kidney cells was significantly decreased, and the nephrotoxicity was reduced as well. Because mice express both Oct1 and Oct2 in the kidney, while in humans OCT2 is the major renal organic cation transporter, *Oct1/2*$^{-/-}$ mice were regarded as a more appropriate animal model. In *Oct1/2*$^{-/-}$ mice, cisplatin urinary excretion was 45% compared to 91% in wild-type animals (Filipski et al. 2009). Compared with wild-type mice, *Oct1/2*$^{-/-}$ mice showed a much milder nephrotoxicity after cisplatin treatment (Ciarimboli et al. 2010).

MATE1, an efflux transporter expressed on the brush-border membrane of renal proximal tubules, mediates secretion of cisplatin into urine. Nakamura et al. found that the accumulation of cisplatin was significantly increased in *Mate1*$^{-/-}$ mice compared with wild-type animals (Nakamura et al. 2010). When given with MATE inhibitor, pyrimethamine (Ito et al. 2010), the nephrotoxicity of cisplatin was enhanced in mice. Overall, co-administration of cisplatin with drugs that are more specific inhibitor of OCT2 may be a useful approach to prevent cisplatin-induced nephrotoxicity as shown by an animal study evaluating imatinib and cisplatin drug interaction (Tanihara et al. 2009). Another strategy is to modify the structure of platinum drugs to identify candidates that are not or minimally transported by OCT2, for example, carboplatin and nedaplatin, which seem having less susceptibility to nephrotoxicity (Yonezawa and Inui 2011).

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Chapter 8
Intestinal Transporter-Associated Drug Absorption and Toxicity

Yaru Xue, Chenhui Ma, Imad Hanna, and Guoyu Pan

Abstract Oral drug administration is the most favorable route of drug administration in the clinic. Intestinal transporters have been shown to play a significant role in the rate and extent of drug absorption of some, but not all, drug molecules. Due to the heterogeneous expression of multiple transporters along the intestine, the preferential absorption sites for drugs may vary significantly. In this chapter, we aim to summarize the current research on the expression, localization, function, and regulation of human intestinal transporters implicated in altering the absorption of low to medium molecular weight drug molecules. The role played by bile acid transport proteins (e.g., ASBT and OST-α/β) is included in the discussion. The synergistic action of intestinal drug metabolism and transport is also discussed. Despite the complicated regulatory factors, the biopharmaceutics drug disposition classification system (BDDCS) put forward by Wu and Benet may help us better predict the effect of transporters on drug absorption. The drug-induced toxicity in the intestine, which may result from drug-drug interaction, gut microbiota, and bile salt toxicity, is also discussed.

Keywords Intestinal transporter · Drug absorption · Bile acid transport · BDDCS · Drug-induced toxicity

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8.1 Introduction

The small intestine is the main organ for the absorption of most nutrients from the diet that are necessary to sustain a living organism. The organ consists of segments with varying lengths and surface areas (duodenum, jejunum, and ileum) which have overlapping roles in the overall absorption process. Intestinal segments are lined by a layer of endothelial cells (enterocytes) organized on villi structures with varying degrees of tightness and act as a barrier between the interior and exterior body chambers. Not surprisingly, these enterocytes when fully matured and differentiated express a wide variety of transporters in the intestine (as shown in Fig. 8.1) that function to facilitate the uptake of essential dietary nutrients and vitamins (Giacomini et al. 2010; Hillgren et al. 2013). Transporters are located on either membrane of the enterocytes facing either the intestinal lumen or the systemic blood circulation. Additionally, the enterocytes also express a number of efflux transporters (predominantly on the apical cell membrane) that are believed to have initially evolved to protect the body from potentially toxic dietary substances. Generally, transporters expressed in enterocytes can be classified as members of the adenosine triphosphate (ATP) binding cassette (ABC) and the solute carrier (SLC) superfamilies, based on their amino acid sequences and the mechanism by which they catalyze the movement of molecules across biological membrane.

The prominent intestinal ABC transporters that are believed to potentially affect drug trafficking are ABCB1 (P-glycoprotein, P-gp, or MDR1), ABCG2 (breast cancer resistance protein, BCRP), and ABCC2 (multidrug resistance-associated protein 2, MRP2). Members of the SLC family of transporters include SLC15A1 (peptide transporter; PEPT1), SLCO2B1 (organic anion transporting polypeptide; OATP2B1), SLC22A1/2 (organic cation transporters; OCT1 and OCT2), SLC22A4/5 (organic cation transporter, novel, type 1 and 2; OCTN1/2), SLC29A4 (monoamine transporter; PMAT), and SLC16A1 (monocarboxylate transporter; MCT1)

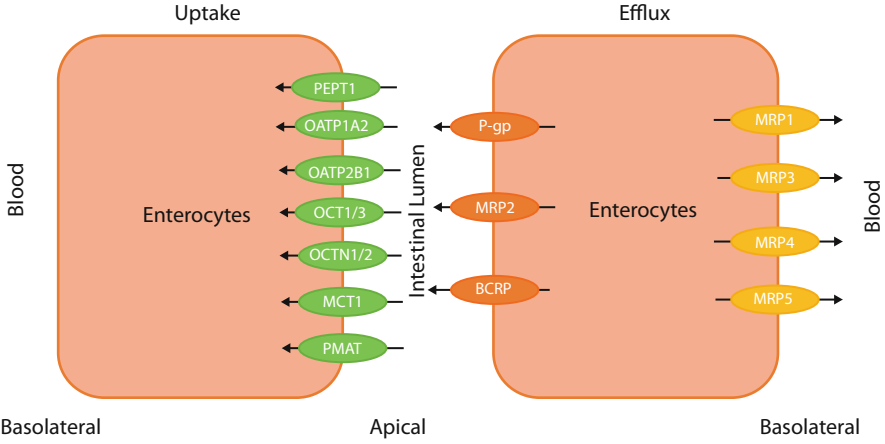

Fig. 8.1 Diagram of major drug transporters expressed in enterocytes. Uptake (left) and efflux (right) transporters localized on the apical (green, orange) and basolateral (yellow) membranes

(Estudante et al. 2013). Transporters demonstrating the highest expression levels (based on mRNA expression) are PEPT1, MCT1, BCRP, MRP2, and P-gp (Hilgendorf et al. 2007; Drozdzik et al. 2018).

The role of intestinal transporters in influencing the oral bioavailability of drug molecules is somewhat controversial. For instance, the demonstration of efficient drug efflux by P-gp or BCRP in an in vitro system in most circumstances does not translate in reduced oral drug absorption. The ability of efflux transport to influence the rate and overall extent of absorption (or bioavailability) depends mostly on the physicochemical properties of individual drug molecules that dictate the aqueous solubility and membrane permeability parameters. The interaction with efflux or uptake transport processes becomes important in those cases where the passive membrane permeability does exceed the rate of active transport in either direction across the enterocyte cell layer (Muller et al. 2017). These physicochemical properties are subject to manipulation by medicinal chemists within a series of compounds in order to improve the absorption potential and possibly the overall systemic exposures. Additional strategies to enhance drug absorption include the use of the high-capacity uptake drug transporters (e.g., PepT1 and ASBT) to deliver drug molecules into the systemic circulation. This approach involves modification of the active drug molecule by conjugating it to established substrates of these uptake transporters in order to facilitate the transport process. The success of this strategy is compound specific and is dependent on whether the pro-drug conjugates are hydrolyzed efficiently upon entry into the systemic circulation to achieve therapeutic levels (Kramer 2011; Zolk and Fromm 2011).

Moreover, the preferential absorption sites for drugs are subject to influence as a result of the heterogeneous expression of transporters along the length of the intestine. In a clinical study involving six subjects, all of whom have no history of gastrointestinal diseases, and Drozdzik et al. 2014 analyzed the gene and protein expression of drug transporters along the human intestine and implicated their functions. Their analysis showed that SLC transporters (especially PEPT1) constituted approximately 67% of the quantified proteins in the small intestine, while the ABC transporters (e.g., MRP2 and MRP3) were mainly localized in the colon, accounting for about 70% of total membrane transport proteins in the tissue. Other members of the ABC family of transporters (e.g., P-gp and BCRP) had higher protein content in the small intestine compared to that in the colon (P-gp, 0.29–1.06 pmol/mg versus 0.15–0.37 pmol/mg; BCRP, 0.19–0.41 pmol/mg versus 0.04–0.16 pmol/mg).

8.2 Roles of Intestinal Transporters in Drug Absorption

8.2.1 P-glycoprotein (P-gp/ABCB1)

P-gp is the most widely investigated intestinal efflux transporter that is often implicated in modulating the kinetics and possibly the extent of drug absorption. This transporter is highly expressed along the apical membrane of enterocytes and is

subject to inhibition and induction by xenobiotics and certain components found in nutrients. The broad substrate specificity of this transporter coupled with its high expression levels enables it to lower the intracellular concentration of transport substrates. Binding of substrates occurs primarily via hydrophobic interactions to the active form of the transporter; substrate access to the active site is via an opening embedded in the inner cell membrane leaflet and the cytosol (Aller et al. 2009). The hydrolysis of ATP and the dimerization of the nucleotide-binding domains induce a broad structural confirmation change resulting in the expulsion of the bound substrate from the cell-cell membrane.

P-gp possesses a broad substrate specificity that includes hydrophobic, neutral, and hydrophilic compounds (Chan et al. 2004; Giacomini et al. 2010). According to the regulatory guidelines and the recommendations of the International Drug Transporter Consortia (Muller et al. 2017), a new molecular entity (NME) is defined as a potential substrate if the efflux ratio (ER) is ≥ 2 in a directional in vitro transport system using polarized cells shown to express adequate amount of P-gp on the apical membrane, i.e.:

$$\mathrm{ER} = P_{\mathrm{app},B-A}/P_{\mathrm{app},A-B} \tag{8.1}$$

where $P_{\text{app-}A}$ and $P_{\text{app, }A-B}$ describe the permeability in basolateral to apical and in apical to basolateral direction, respectively.

In addition to catalyzing the efflux of orally administered compounds, P-gp can also affect parenterally administrated drugs, such as the intravenously administered drug vinblastine (Kunta and Sinko 2004; Muller and Fromm 2011). Access to the transporter in these cases would be either via an interaction with P-gp expressed at the hepatocyte cancalicular membrane (biliary secretion) or the intestine (direct intestinal secretion).

P-gp expression can be induced by various factors, including clinical drugs, environmental xenobiotics, and dietary compounds (Estudante et al. 2013). For example, as a potent P-gp inducer, rifampin could significantly decrease the exposure of P-gp substrates, such as digoxin (Greiner et al. 1999) and talinolol (Westphal et al. 2000). In the healthy volunteers, significant reductions in digoxin AUC_{0-3} (30%), AUC_{0-24} (25%), and $C_{\max}$ (38%) were found following oral administration of rifampin (300 mg twice daily, for 7 days). Another clinical study revealed that oral concomitant rifampin (600 mg per day for 9 days) therapy significantly upregulated expression of P-gp mRNA (2.41-fold) and protein (4.20-fold) in the duodenal biopsy specimens, which were well correlated to the increased systemic clearance of talinolol. Most P-gp inhibitors exert their function by altering ATP hydrolysis pathway, P-gp expression, or competing for a binding site. In clinic, it was found that P-gp inhibitors may increase their substrate plasma exposure significantly. For example, co-administration of an efficacious inhibitor of P-gp, cyclosporine, strongly enhanced the systemic exposure of docetaxel (7.3-fold increase) (Malingre et al. 2001b) and paclitaxel (8.3-fold) (Meerum Terwogt et al. 1999) upon oral administration.

Numerous ingredients from fruits, vegetables, and herbs can interfere the activity of P-gp and may cause detrimental effects on drug pharmacokinetics (Murakami and Takano 2008; Chen et al. 2012). One of the most recognized interactions is the ingestion of grapefruit juice, which can change drug absorption not only by inhibiting CYP3A enzymes (Bailey et al. 1998) but also by inhibiting P-gp-mediated intestinal absorption (Benet 2009). Such interactions have been documented for talinolol (Spahn-Langguth and Langguth 2001) and fexofenadine following both in vitro and in vivo studies. Other natural P-gp inhibitors have been identified such as alkaloids, flavonoids, coumarins, resins, saponins, and terpenoids (Foster et al. 2001). This suggested that systemic exposure of P-gp substrates could be altered in a manner similar to that observed with grapefruit juice intake. However, the extent of this interaction and the potential for taking advantage of it as a therapeutic endpoint is limited due to the higher inter-individual difference in the extent of P-gp expression among other physiological factors.

Several studies have reported that the mRNA and protein abundance of P-gp increase from proximal to distal small intestine (Fojo et al. 1987; Stephens et al. 2001; Mouly and Paine 2003b; Muller et al. 2017). The ascending pattern of P-gp expression in the small intestinal tract suggests that non-CYP3A-metabolized P-gp substrates would have appreciably different effective permeability values along the length of the intestine (i.e., proximal versus distal). This hypothesis was verified by the intestinal pharmacokinetic profiles of talinolol, paclitaxel, and digoxin. In the intestinal catheter perfusion studies, the perfusion rate of talinolol was higher in the distal region than that in the proximal site of the human intestine (Gramatte et al. 1996). Similarly, increased absorption of paclitaxel and digoxin along the *mdr1a*$^{-/-}$intestine was site-dependent, with higher changes in the ileum and distal colon than in the jejunum and proximal colon (Stephens et al. 2002).

8.2.2 Breast Cancer Resistance Protein (BCRP; ABCG2)

BCRP is another efflux transporter expressed at the apical surface of enterocytes, where it plays a role in limiting drug bioavailability as well as protecting tissues against the toxicity of endogenous or exogenous toxins (Adachi et al. 2005). Unlike P-gp, the protein abundance of BCRP does not vary significantly along the length of the human intestine (Takano et al. 2006; Bruyere et al. 2010), although a recent study (Drozdzik et al. 2014) suggested higher BCRP expression in the distant intestine compared to proximal. The results of this study were put into question when the larger intersubject variability in the expression is taken into consideration. BCRP also possesses a diverse range of substrates that includes hydrophobic and hydrophilic substrates (Ni et al. 2010). Physiologically, BCRP also regulates the intracellular concentrations of important biological cofactors and prosthetic groups such as heme, porphyrins, riboflavin, and estrogens (Grube et al. 2007; Krishnamurthy et al. 2007; van Herwaarden et al. 2007). The most investigated BCRP substrates includes anticancer agents, hypotensive drugs, antiviral agents, and natural products.

Numerous reports have demonstrated that BCRP plays a vital role in limiting drug absorption across the intestine in a synergistic manner with P-gp and MRP2. Of the BCRP substrates, many anticancer agents (e.g., doxorubicin, topotecan, methotrexate, tyrosine-kinase inhibitors imatinib, nilotinib, and dasatinib) as well as some natural products (e.g., quercetin and genistein) are also P-gp substrates (Arimori et al. 2003; Dohse et al. 2010; Ohura et al. 2011). Dual BCRP and MRP2 substrates include estradiol-17β-glucuronide, resveratrol glucuronide, naringenin glucuronides, methotrexate, SN 38 and SN 38 glucuronide, and ezetimibe-glucuronide (Dahan and Amidon 2009; Vlaming et al. 2009; Xu et al. 2009; Juan et al. 2010).

Few reports about BCRP induction have been published. In vitro studies indicated that the pregnane X receptor (PXR) ligand rifampicin and glitazones (rosiglitazone and troglitazone) can significantly induced the mRNA expression of ABCG2 in human hepatocytes in a dose-dependent fashion (Weiss et al. 2009). However, the effect of BCRP induction in vivo has only been detected in animal models but not in clinic. One case in rat showed that oral administration of efavirenz (20 mg/kg for 5 days) upregulated the intestinal expression of Abcg2 which was accompanied by the decline intestinal absorption of the drug itself (Peroni et al. 2011). Another study in broilers showed that rifampicin upregulated the level of P-gp and BCRP in small intestine, thus limiting the orally bioavailability of enrofloxacin (Guo et al. 2016). Despite these, it is difficult to definitively implicate BCRP in altering the pharmacokinetics of select substrates due to considerable involvement of additional transport or metabolic clearance pathways.

Potent BCRP inhibitors have a wide range of chemical structures spanning from steroid hormones (17β-estradiol and 17β-estradiol-3-sulfate) (Imai et al. 2002; Suzuki et al. 2003), antiviral agents (abacavir, amprenavir, atazanavir, nelfinavir, saquinavir, delavirdine, and efavirenz) (Weiss et al. 2007), statins (atorvastatin, cerivastatin, fluvastatin, pitavastatin, rosuvastatin, and simvastatin) (Hirano et al. 2005), and anticancer agents (erlotinib, nilotinib, and elacridar) (Tiwari et al. 2009). In certain cases, BCRP inhibition has been recognized as potential method to improve the oral drug bioavailability with the help of excipients, such as Pluronic P85 and Tween 20 (Yamagata et al. 2007).

8.2.3 Multidrug Resistance-Associated Protein Families (MRPs; ABCCs)

Up to date, nine members of MRP family have been identified, among which only MRPs 1–5 were shown to be capable of drug transport (Yazdanian et al. 1998; Yu et al. 2007; Liu et al. 2010). In particular, apically expressed MRP2has been implicated in reducing the rate of intestinal absorption of select compounds or compound classes. The other four MRPs are believed to be localized on the basolateral membrane of the enterocytes and can participate in the overall drug transport process either into or out of the enterocyte cell layer.

MRP2 have been reported to transport several endogenous compounds like leukotrienes, bile acids, and glutathione conjugates, in addition to multiple drug metabolites conjugated to glucuronic acid or sulfate (Liu et al. 2010). In cooperation with P-gp and BCRP, MRP2 can also modulate the pharmacokinetics of multiple drugs, especially the unconjugated organic anions such as methotrexate (El-Sheikh et al. 2007), vinblastine (Tang et al. 2002), irinotecan and its metabolite SN-38 (Chu et al. 1998), pravastatin, ceftriaxone, and ampicillin (Payen et al. 2002). Other substrates include angiotensin II AT1 receptor antagonists (e.g., valsartan, olmesartan) (Yamashiro et al. 2006; Yamada et al. 2007), angiotensin converting enzyme inhibitors (ACEIs, e.g., fosinopril) (Green and Bain 2013), fexofenadine (Akamine et al. 2010), and lopinavir (van Waterschoot et al. 2010).

Estimation of the MRP2 and MRP3 abundance along the human gastrointestinal tract demonstrated relatively higher levels of expression in colonic tissues compared to other GI segments (Drozdzik et al. 2014; Muller et al. 2017). Particularly, the expression value came as a surprise since the estimated MRP3 level comprised more than one-third of the total transporter protein abundance in the colon. It was therefore assumed that MRP3, owing to its cellular localization on the basolateral membrane of enterocytes, may play a significant role in the reentry of certain substrates back into the circulation (Deeley et al. 2006; Kitamura et al. 2010). This finding suggested that the contribution of MRP3 to overall drug absorption and disposition is poorly understood (Jia et al. 2014; Keppler 2014).

8.2.4 Solute Carrier Transporters (SLC; SLCO)

Several members of the solute carrier family of proteins are expressed along the length of the intestinal tract. The transport mechanism(s) varies significantly among the various members of this class. These mechanisms include direct exchange of organic cations (OATs), co-transport down sodium (ASBT), or proton (PepT1) gradients. The peptide transporter (PepT1) is a highly expressed intestinal transporter that is responsible for the uptake of amino acids/peptides as nutrients from dietary sources. The protein abundance of Pept1 is the highest of all investigated transporters in the small intestine and accounts for approximately 50% of the total transport protein abundance (2.63–4.89 pmol/mg) (Drozdzik et al. 2014). The catalytic activity is driven by the difference in the pH value of the intestine (~6.0–6.5) to that in the systemic circulation (pH 7.2–7.4). As a result, the rate of PepT1 transport is not directly proportional to the expression level due to the non-uniform pH along the length of the intestine (Guo et al. 2012). The Pept1-mediated transport (uptake) activity is highest in the duodenum/proximal jejunum, where the pH is slightly acidic and tends to be lower at distal segments, possibly as a result of the increase in the luminal pH value (Smith et al. 2013).

PepT1 plays a major role in nitrogen supply to the body by mediating intestinal absorption of di- and tripeptides. The key role for PepT1 in the intestinal uptake of peptides was confirmed in Pept1 null mice after oral and intravenous dipeptide

glycylsarcosine (Gly-Sar) dosing. Plasma concentrations of glycylsarcosine are similar in wild-type and PepT1 null mice after intravenous administration but were appreciably (~50%) reduced in the null mice after oral administration (Hu et al. 2008b).

Beyond food-derived di- and tripeptides, PepT1 has been of great pharmacological and pharmaceutical relevance that it also accepts many drugs, prodrugs, and drug candidates. For example, PepT1 has been unequivocally established as the major player in the intestinal absorption of β-lactam antibiotics, ACE inhibitors, and antiviral drugs (Brandsch 2013).

The PEPT1/Pept1 expression and activity can be influenced by food intake, fasting, and disease. Several investigations have been done to examine the effect of PepT1 on drug-food interactions, particularly the co-administration of milk and PEPT1 substrates. For example, milk intake could markedly limit the absorption of oseltamivir, a PepT1 substrate, in rat (Ogihara et al. 2009). However, in humans, this inhibition was only limited to the initial absorption rate but not the total extent of oseltamivir absorption (Morimoto et al. 2011). The influence of fasting on Pept1 expression was studied in mice. It was reported that 16 h of fasting could significantly increase the expression of Pept1 in the intestine, which corresponded with the improved to oral absorption of Gly-Sar (Ma et al. 2012).

Some diseases can alter Pept1 expression significantly, and several reports suggested that PEPT1 activity and expression may be correlated with systemic insulin levels, which were deficient in the type 1 diabetics (decreased PEPT1 activity) and excessive in the hyperinsulinemia (increased PepT1 activity) (Thamotharan et al. 1999; Watanabe et al. 2003; Bikhazi et al. 2004; Hindlet et al. 2009). PEPT1 (at least in the colon) was also activated during inflammatory and immune responses, which was hypothesized to further aggravate the outcomes of such conditions. These studies showed that di-/tripeptides of enterobacteria could be transported by Pept1 and consequently bind to the innate immune receptors resulting in the activation of downstream immunogenic responses (Ingersoll et al. 2012).

8.2.5 Organic Cation Transporters (OCT, OCTN; SLC22A)

Human SLC22A family consists of the electrogenic cation transporters OCT1-3/SLC22A1-3, the organic cation/carnitine transporters OCTN1-2/SLC22A4-5, and OCT6/SLC22A16 (Koepsell et al. 2007). In the intestine, the localization of OCT1 is controversial. Initially, OCT1 expression was believed to localize to the basolateral membranes of the enterocytes. However, since the year 2013, more and more evidences support the idea that OCT1 is localized on the apical side of human and mice enterocytes (Han et al. 2013; Muller et al. 2017; Mendes et al. 2018). OCTN1, OCTN2, and OCT3 are expressed at the enterocyte apical membrane (Klaassen and Aleksunes 2010). OCT2 and OCT6 are not detected in the intestine (Koepsell et al. 2007).

According to the clinical study about the protein abundance of multidrug transporters along the human intestine (Drozdzik et al. 2014), OCT1 was shown to be homogeneously distributed along the entire human intestine, accounting for approximately 10% of the total transporter protein abundance. In contrast, OCT3 expression was found to constitute only approximately 1% of the total abundance. The reported drugs that are transported by human OCT1 include morphine (Tzvetkov et al. 2013), acyclovir, ganciclovir (Takeda et al. 2002), and metformin (Shu et al. 2007). OCT1 has a large range of inhibitors, which gives rise to the high possibility of DDI. Potent OCT1 inhibitors include atropine, butylscopolamine, clonidine, diphenhydramine, quinine, ranitidine (Muller et al. 2005), memantine (Amphoux et al. 2006), midazolam (Zhang et al. 1998), and rosiglitazone (Bachmakov et al. 2008). Nonetheless, there are a few reports that linked intestinal OCT1-mediated transport to clinically relevant DDIs. An example of such a potential interaction is the effect of the antituberculosis drug ethambutol on the pharmacokinetics of lamivudine or metformin (Pan et al. 2013). This interaction is postulated to occur due to the inhibition of intestinal hOCT1 and hOCT3 by ethambutol. The majority of OCT-mediated interactions are attributed to interference with renal and hepatic OCT-mediated transport.

OCTN1/SLC22A4 and OCTN2/SLC22A5 can transport carnitine in addition to a number of organic cations. Typical OCTN1 substrates include mitoxantrone and doxorubicin (Okabe et al. 2008), tetraethylammonium, verapamil, quinidine (Yabuuchi et al. 1999), and ergothioneine (Grundemann et al. 2005). OCTN2 is known for its efficient transport of carnitines (D-isomer, $K_m = 10.9$ μM; L-isomer, $K_m = 4.3$ μM). Prolonged administration of OCTN2 inhibitor, such as emetine (Wagner et al. 2000), can induce systemic carnitine deficiency. Other substrates of OCTN2 include β-lactam antibiotics cephaloridine (Ganapathy et al. 2000), sorafenib, sunitinib (Hu et al. 2009), and imatinib (Hu et al. 2008a).

The expression of OCTN1 and OCTN2 gene is subject to modulation as a result of intestinal disorders which include inflammatory bowel disease. Under diseased conditions, the release of cytokines such as IFN-γ and TNF-α have been postulated to enhance OCTN2 expression in the intestinal tract especially in the colonic segments (Fujiya et al. 2011). However, in a case of ulcerative colitis, OCTN1 and OCTN2 were downregulated (Yamamoto-Furusho et al. 2011). Interestingly, the frequency of OCTN variants (*SLC22A4 1672C>T* and *SLC22A5 207G>C*) was higher in Crohn's disease (CD) patients compared to that in healthy groups (Babusukumar et al. 2006; Bene et al. 2006), suggesting a possible association of higher CD susceptibility in carriers of these OCTN variants.

8.2.6 Organic Anion Transporter Polypeptide (OATP; SLCO)

At least 39 members of the OATP superfamily have been identified to date. Most of the OATP transporters whose activity has been examined have been shown to be widely expressed throughout the body including organs such as the liver, kidney,

intestine, heart, brain, and placenta (Varma et al. 2010). The OATPs of interest from a clinical perspective include hepatic OATP1B1 and OATP1B3 and intestinal OATP2B1 and OATP1A2 (Kobayashi et al. 2003; Glaeser et al. 2007). OATP2B1 is expressed in a wide variety of tissues (including the liver and skeletal muscles); however, its expression in the intestine has received the most amount of attention due to its potential to impact the absorption of select drug molecules (Tamai et al. 2000; Meier et al. 2007). Similarly, OATP1A2 activity in the intestine has been suggested; however, follow-up investigations of the expression of active OATP1A2 could not be demonstrated with certainty (Glaeser et al. 2007). Moreover, the lack expression of OATP1A2 in the human intestine were in line with the clinical study in 2014 (Drozdzik et al. 2014), where no OATP1A2 gene or protein was detected in the human intestine. The expression of most OATP transporters is localized to the basolateral membrane of barrier-function cells acting to facilitate the entry of solutes into these cells for downstream processing. Conversely, in human enterocytes, OATP2B1 expression is localized to the apical membrane (Kobayashi et al. 2003). Moreover, OATP2B1 appears to possess a narrow substrate specificity (Nozawa et al. 2004) when compared to that of other members of the SLCO superfamily. In vitro studies have identified that OATP2B1 is capable of transporting estrone-3-sulfate, bromsulphthalein, HMG-CoA reductase inhibitors (statins, atorvastatin, cerivastatin, and simvastatin) (Kobayashi et al. 2003; Grube et al. 2006; Hirano et al. 2006; Visentin et al. 2012), glyburide (Satoh et al. 2005), and telmisartan glucuronide (Ishiguro et al. 2008). The inhibition of OATP2B1 by substances present in nutrients of xenobiotics and their metabolites has been implicated with alterations in the pharmacokinetics of co-administered compounds, therapeutics, and/or their metabolites (Yu et al. 2017). Examples of xenobiotics capable of inhibiting OATP2B1 include members of the statin family of drugs (atorvastatin, cerivastatin, and simvastatin) (Grube et al. 2006), cyclosporine, rifampicin, paclitaxel, montelukast, silibinin hemisuccinate (Ho et al. 2006; Letschert et al. 2006), ritonavir, saquinavir, and rosiglitazone (Annaert et al. 2010). Additionally, component found in many nutritional supplements and fruit juices have been shown to influence OATP2B1 activity. These agents include component found in herbal medications (Kashihara et al. 2017), fruit juice (apple juice, grapefruit juice, orange juice, and pomelo juice), and green tea. Changes in the pharmacokinetics of select OATP2B1 substrates caused by the ingestion of several fruit juices have been widely investigated. The verification of the clinical significance of intestinal OATP2B1 activity is often challenging since the same agents used to examine potential effect on intestinal OATP2B1 activity often interact with the activity of intestinal drug-metabolizing enzymes and transporters including CYP3A4 and P-glycoprotein, respectively.

Examples of this situation include the inhibitory effect on many fruit juices on CYP3A4 and P-glycoprotein activity which would be expected to increase the bioavailability of respective substrates (Chayen and Rosenthal 1991; Takanaga et al. 1998). Fexofenadine represents an interesting case where a 63% decrease in its systemic exposure is observed when co-administered with grapefruit juice. This suggested that the contribution of OATP-mediated fexofenadine uptake activity significantly contributes to its systemic exposure. Moreover, the uptake activity

appears to overcome the apparent efflux activity by P-gp as it has been shown to be capable of fexofenadine transport as well (Imanaga et al. 2011). The ingredients in grapefruit juice responsible for the inhibition of drug-metabolizing enzymes and transporters were reported to be the furanocoumarin bergamottin and naringin, respectively (de Castro et al. 2007; Shirasaka et al. 2010). The co-administration of fruit juices or green tea has also been shown to reduce the systemic exposure of the cardiovascular agents (atenolol, celiprolol, and nadolol) by about 85% (Lilja et al. 2003; Lilja et al. 2004; Jeon et al. 2013).

Owing to the tissue localization of OATP2B1 (and possibly OATP1A2), its activity can theoretically be exploited to enhance the absorption of biologically active molecules in a manner analogous to that taking advantage of PepT1-mediated transport. PepT1 activity has been extensively explored as a tool for oral drug delivery by conjugating potential drug to amino acid residues (Balimane et al. 1998; Tamai et al. 1998; Nozawa et al. 2003; Azevedo and Pashkuleva 2015). An example of a potential OATP-aided drug absorption was documented with tebipenem-pivoxyl which acts as a prodrug for tebipenem, a carbapenem antibiotic. The active principle in this case exhibited high bioavailability which was postulated to be due to OATP2B1- and possibly OATP1A2-mediated uptake and not that of PepT1 (Tamai 2012). The diverse structural requirements exhibited by members of the OATP family, and OATP2B1 in particular, make the task of utilizing this activity to enhance drug bioavailability rather challenging. In summary, OATP2B1 and not OATP1A2 has potentially affect the absorption of select drug molecules, and the inhibition of intestinal OATP2B1 activity may result in decreased systemic bioavailability of select transported substrates.

8.2.7 Plasma Membrane Monoamine Transporter (PMAT; SLC29A4)

Plasma membrane monoamine transporter (PMAT/SLC29A4) is a relatively new organic cation transporter, which has the highest expression in human brain (Engel et al. 2004). PAMT protein expression was also confirmed in the human intestine (Zhou et al. 2007), as well as in the choroid plexus, cerebellum, kidney, and heart (Barnes et al. 2006; Xia et al. 2009; Duan and Wang 2013). PMAT belongs to the family of equilibrative nucleoside transporters (ENTs), which is encoded by the SLC29 gene family (Acimovic and Coe 2002). Different from other members of this subfamily (viz., ENT1–3), PMAT (also referred to as ENT4) does not interact with nucleosides or their structural analogs. Instead, PMAT transports monoamine neurotransmitters, such as serotonin (5-HT), dopamine (DA), neurotoxin 1-methyl-4-phenylpyridinium (MPP^+), guanidine, histamine, epinephrine, and norepinephrine (Engel et al. 2004; Engel and Wang 2005). Moreover, PMAT also shares considerable substrate and inhibitor selectivity with members of the human OCT family. For example, PMAT has been shown to participate in the intestinal absorption of metformin and atenolol (Zhou et al. 2007; Mimura et al. 2017), and the demonstrated

PMAT inhibitors have also been shown to inhibit human OCT although the inhibitors investigated tend to generally exhibit poor selectivity. One of the most potent and selective inhibitors of PMAT is lopinavir, which exerts more than 120-fold selectivity toward PMAT compared to OCT1 (Wang 2016). Overall, the data on PMAT activity in the human intestine and its relevance to the absorption of small drug molecules and the potential for drug interaction is rather limited and requires further verification.

8.2.8 Monocarboxylate Transporter 1 (MCT1; SLC16A1)

Monocarboxylate transporters (MCTs) are composed of 14 isoforms, all encoded by the SLC16A family (Jones and Morris 2016). The first discovered member, MCT1/SLC16A1, is well characterized and highly expressed on the apical side of small intestine (Iwanaga and Kishimoto 2015). Transporters in this class are believed to facilitate the intestinal drug absorption of low permeability anionic compounds along with PepT1 and members of the OATP family. Key substrates of MCT1 include monocarboxylates like lactate, pyruvate, and ketone bodies (Varma et al. 2010). Other MCT1 substrates include exogenous acids (e.g., benzoic acid, p-aminohippuric acid, salicylic acid, valsartan, mevalonic acid, and foscarnet) and the β-lactam antibiotics (e.g., carbenicillin indanyl sodium, phenethicillin, propicillin) (Jones and Morris 2016).

8.3 Regulation of Intestinal Transporters

Numerous factors can affect the expression, function, and localization of intestinal transporters at the transcriptional and post-transcriptional levels. The transcriptional mediators include nuclear receptors which can target the genes of most transporters. The post-transcriptional regulation can affect the protein expression, localization, and function of transporters without altering the mRNA expression. Different factors under the physiological or pathophysiological conditions have also been demonstrated to exert considerable effect on the expression and activity of intestinal transporters.

8.3.1 Transcriptional Regulation

The transcriptional regulation of intestinal transporters is mediated by nuclear receptors such as the pregnane X receptor (PXR, NR1I2), constitutive androstane receptor (CAR, NR1I3), farnesoid X receptor (FXR, NR1H4), vitamin D receptor (VDR, NR1I1), and peroxisome proliferator-activated receptor (PPAR, NR1C1). These nuclear receptors exert their functions by hetero-dimerizing with the retinoid

X receptor α (RXRα, NR2B1) and bind to sequences in the response elements (Klaassen and Aleksunes 2010) resulting in the activation of specific ligands.

The mostly studied intestinal transporter MDR1 (P-gp) has been reported to be regulated by PXR, CAR, and VDR. Initially, P-gp expression and activity were found to be influenced following PXR activation with rifampin (Greiner et al. 1999; Magnarin et al. 2004). Later, it was reported that both PXR and CAR could regulate the *ABCB1* gene in the intestine by targeting the upstream promoter element (Geick et al. 2001; Burk et al. 2005). The treatment of the natural ligand of the vitamin D receptor (VDR), 1α,25-dihydroxyvitamin D3 (1, 25 (OH)2D3) also induced the expression of P-gp in the human intestinal or colonic cell lines (Schmiedlin-Ren et al. 1997; Aiba et al. 2005). This activation involved in multiple functional VDR response elements in the promoter of the human *ABCB1* gene (Saeki et al. 2008).

MRP2 is also regulated by several nuclear receptors, especially PXR, CAR, and FXR. Studies demonstrated that MRP2 could be regulated by PXR, CAR, and FXR dependent on a common response element ER-8 in the 5′-flanking region of the gene (Kast et al. 2002). Whereas the effect of VDR on P-gp activation is uncertain, 1,25 (OH)2D3 has been shown to effectively induce the expression of rat intestinal Mrp2 (Chow et al. 2010; Maeng et al. 2011). Additionally, VDR activation has also been shown to contribute to the induction of rat intestinal PepT1, Mrp3, and Mrp4.

The ABCG2 gene and BCRP protein expression and catalytic activity in human intestinal cells could be induced by aryl hydrocarbon receptor (AHR) ligands (tetrachlorodibenzo-p-dioxin, dimethyl-benzo(*a*)pyrene, and 3-methylcholanthrene). AHR was shown to be the direct transcriptional regulator using a combination of gene promoter DNA sequence analysis, chromatin immunoprecipitation, and electrophoretic mobility shift assays (Tan et al. 2010).

OCTN2 has long been reported to be regulated by PPARα. For example, PPARα agonist clofibrate could upregulate expression of Octn2 in the small intestine of animal models (van Vlies et al. 2007; Ringseis et al. 2008a, b), leading to increased absorption of carnitine from diet. And the increased absorption of carnitine from the intestine contributed to increased carnitine concentrations in the liver and other tissues. Later, it was further demonstrated that regulation of *SLC22A5* gene by PPARα was highly conserved across species, indicating that human *SLC22A5* genes are also PPARα target genes (Luo et al. 2014). PPARα and PPARγ agonists could transcriptionally increase the *Slc22a1* gene expression, which accounted for the enhanced cellular organic cation uptake (Nie et al. 2005).

The transcriptional regulation of PepT1 is distinct from other transporters. Sp1 was found to be a basal transcriptional regulator of human PepT1 (Shimakura et al. 2005). And Sp1 may exert its function by interacting with another transcription factor Cdx2 (Shimakura et al. 2006).

8.3.2 Posttranscriptional Regulation

The posttranscriptional regulation can be classified into three processes: posttranscriptional (modifications in transport or processing of mRNA), translational

(modulation of protein synthesis), or posttranslational (changes in protein stability or localization or substrate affinity) regulation. Through the research on PubMed, we found that the posttranscriptional regulation of MRP2 was well studied. And we choose to take MRP2 as an example to elucidate the mechanism of posttranscriptional regulation of intestinal transporters.

For instance, posttranscriptional regulation by mRNA splicing was shown to be one of major causes of truncated or nonfunctional proteins in Dubin-Johnson syndrome (DJS) (Tate et al. 2002; Mor-Cohen et al. 2005). Translational regulation of rat Mrp2 protein could be related to PXR activation, which does not always lead to the transcriptional regulation. It was reported that PCN treatment could increase the protein expression of liver Mrp2 without changing the mRNA level (Jones et al. 2005).

In rat intestinal epithelia, it was found that a conventional PKC activator thymeleatoxin reduced activity of Mrp2, accompanied by decreases in Mrp2 expression in the brush border membrane of the small intestine without affecting total expression of Mrp2 protein, and the interaction between Mrp2 and ezrin was demonstrated by immunoprecipitation analysis. These results indicate that PKC activation could interfere with the interaction between Mrp2 and Ezrin, leading to the internalization of rat Mrp2 (Nakano et al. 2009).

8.3.3 Physiological Conditions

8.3.3.1 Diurnal Rhythm

The influence of diurnal rhythmicity on the expression of drug transporters has been assessed in many studies. And this diurnal variation in intestinal drug transporters may contribute to circadian rhythmicity in drug pharmacokinetics. It was observed that the intestinal transporters, Pept1, Mrp2, P-gp, Bcrp, and Mct1, could be significantly affected by diurnal rhythmicity. Pept1 activity was found to be different between the dark and the light phases (Pan et al. 2002). And the mechanism was supposed to be in the transcriptional level due to the synchronized change in the protein and mRNA expression. The expression of intestinal Pept1 in rat was the highest in the morning hours and the lowest 12 h later. P-gp, Mct1, Mrp2, and Bcrp expression levels in rat jejunum were also shown to vary during the day, with 1.6–5.4-fold change between the maximum and the minimum levels (Stearns et al. 2008). The degree of translation of these findings from rodents to humans is somewhat limited due to the nocturnal habits of rodent. Finally, most of these studies examined the transcriptional activation of these transporters, and few studies examined the downstream activity.

8.3.3.2 Gender and Hormonal Regulation

In pregnant mice, steroid hormones (progesterone) could downregulate the P-gp, Mrp2, and Bcrp mRNA expression, possibly by reducing the PXR/Pxr signaling (Moscovitz et al. 2017). This result was verified by an in vitro study in intestinal LS174T adenocarcinoma cells, where progesterone reduced P-gp and MRP2 mRNA expression by 30–40%. PepT1 expression has also been shown to be sensitive to hormone treatments such as insulin and thyroid hormone. In particular, insulin was shown to promote the trafficking of Pept1 from the cytosol to the membrane, thereby increasing the measured uptake activity without a concomitant change in the rate of PepT1 protein synthesis (Thamotharan et al. 1999). Conversely, thyroid hormone (specifically triiodothyronine) was shown to reduce Gly-Sar uptake activity in Caco-2 cells, possibly by decreasing the transcription and/or stability of PepT1 mRNA (Ashida et al. 2002).

8.3.3.3 Genetic Polymorphism

Genetic polymorphism is an unavoidable factor that can influence the expression of drug transporters under physiological conditions. Single nucleotide polymorphisms (SNPs) appear frequently in human genome and account for about 90% human genetic mutations (Chaudhary et al. 2015). SNPs present in intestinal transporters have widespread effects ranging from effects on oral bioavailability, drug resistance, and susceptibility to certain diseases.

A number of SNPs have been described in the *ABCB1*gene encoding P-gp. The three most reported SNPs are C1236T in exon 12, G2677 T/A in exon 21, and C3435T in exon26 (Brambila-Tapia 2013). Numerous studies suggested that mutations in the *ABCB1* gene can affect the pharmacokinetics and pharmacodynamics of P-gp substrates. For instance, the oral exposure of P-gp substrate levosulpiride was significantly higher in the subjects with *ABCB1 2677TT* and *3435TT* mutations (Cho et al. 2010). Similarly, the oral bioavailability of digoxin in *G/G2677C/C3435* subjects was also increased (Kurata et al. 2002) likely due to decreased P-gp-mediated efflux.

A number of genetic polymorphisms in the ABCC1 gene encoding MRP2 have also been described (Arana et al. 2016). However the effect that these mutations exert on gene expression or MRP2 function has not been fully elucidated. Evidence showing an effect of combined polymorphisms of *ABCB1 C1236T*, *ABCB1 C3435T*, and *ABCC2 C24T* on fexofenadine pharmacokinetics have been described (Akamine et al. 2010). The *ABCC2 C24T* polymorphism alone did not appear to have an effect on the expression of human MRP2 or the intestinal absorption of MRP2 substrate talinolol (Bernsdorf et al. 2006).

Genetic polymorphisms of the BCRP gene appear to play an important role in the pharmacokinetics of multiple BCRP substrates. Patients with *ABCG2 C15994T* variants possessed a higher clearance of imatinib (Poonkuzhali et al. 2008) and had a 67% chance to develop gefitinib-dependent diarrhea (Lemos et al. 2011) compared to the wild-type individuals. Pharmacokinetics of some HMG-CoA reductase inhibitors, such as rosuvastatin (Zhang et al. 2006) and atorvastatin (Tsamandouras et al. 2017), could be significantly altered by BCRP mutations, but others like pravastatin (Ho et al. 2007; Kivisto and Niemi 2007) and pitavastatin (Ieiri et al. 2007) were little affected.

Reports on the polymorphism of transporters in SLC family generally focused on OATPs. A SNP of *OATP2B1*, *OATP2B1∗3*(*S486F*), was found in ~31% of the Japanese subjects and resulted in more than 50% reduction of the transport capacity (Nozawa et al. 2002). Similar to OATP2B1, genetic variations of OATP1A2 also play a role in the substrate absorption. In vitro studies showed that two *OATP1A2* variants (A516C and A404T variants) markedly reduced the uptake of OATP1A2 substrates estrone-3-sulfate and two delta-opioid receptor agonists (Lee et al. 2005). However as mentioned previously, the intestinal expression of OATP1A2 is not believed to be high enough to result in meaningful changes in rate or extent of absorption of substrates that are subject to transport by this transporter.

8.3.3.4 Pathophysiological Conditions

PepT1 is one of the most reported intestinal transporters easily affected by diseases, such as diabetes, intestinal resection or transplant, and inflammatory bowel diseases (IBDs). Uncontrolled diabetes could upregulate expression of Pept1 mRNA and protein both in the intestine and the kidney without affecting the gene transcriptional rate (Gangopadhyay et al. 2002). The regulatory mechanisms controlling Pept1 expression/activity after intestinal resection or transplantation are rather complex posing significant challenges to therapeutic management and outcomes. In experimental animals with resection of the proximal intestine (Avissar et al. 2001) as well as in humans with short-bowel syndrome (SBS), the expression of Pept1/PEPT1 protein or mRNA expression showed no significant changes in the ileum but increased in the colon (Ziegler et al. 2002). The expression of Pept1 after intestinal transplant was more contradictory between different studies (Watson et al. 1988; Motohashi et al. 2001). Furthermore, patients with IBDs, such as ulcerative colitis and Crohn's disease, may have induction of the PEPT1 expression in their colon, where no PEPT1 was detected in healthy individuals (Merlin et al. 2001). Reasons for the PEPT1 induction in the colon of patients with IBDs remain unknown. A hypothesis is that the alterations may be attributed to release of inflammatory mediators during the IBDs. Inflammatory cytokines such as TNF-α and IFN-γ were shown to increase PEPT1/PepT1 expression and activity in the Caco-2 cells and in mouse proximal and distal colon but not in jejunum or ileum (Vavricka et al. 2006).

Apart from PEPT1, numerous drug transporters can be altered during infection and inflammation (Petrovic et al. 2007). These changes in the expression or activity of intestinal transporters could significantly impact the pharmacokinetics and dynamics of the substrates. The major three ABC efflux transporters P-gp, MRP2, and BCRP were all downregulated by the inflammation in the intestine. Inhibition of the mRNA, protein, or activity of the three transporters increased the blood concentration of relevant substrates. For example, in patients with ulcerative colitis, BCRP and P-gp expression was strongly reduced, which was negatively correlated with the levels of IL-6 mRNA (Englund et al. 2007). Inflammation was also found in patients with obstructive cholestasis, and the downregulation of MRP2 expression in the duodenum was supposed to be regulated by IL-1β (Dietrich et al. 2004).

8.4 Synergistic Action of Transporters

8.4.1 Synergistic Action of P-glycoprotein and CYP3A4

Despite the abundant P-gp expressed in the apical membrane of the small intestine, the intestinal tract also contains many cytochrome-metabolizing enzymes including CYP450 isoforms (Nakanishi et al. 2010; Abuasal et al. 2012). Among them, CYP3A4 is the most prominent drug-metabolizing enzyme present in the human intestine which adds an additional dimension to the understanding of potential drug-drug interactions and in vivo to in vitro extrapolation since it shares considerable subrate selectivity with P-gp (Kalitsky-Szirtes et al. 2004; Abuasal et al. 2012). In the enterocytes, owing to the spatial relationship of CYP3A4 and P-gp, repeated exposure of substrate drugs to metabolism by CYP3A4 may be possible and is regulated by passive absorption and active P-gp efflux (Zhao et al. 2013).

Previous studies have been conducted to characterize the potential interaction between P-gp and CYP3A4 at the level of the intestine. P-gp-mediated efflux in the intestine is postulated to act as a way to increase the tissue residence time due to the repeated entry and exit of the transport and metabolism susceptible drug molecules. The end result of this interaction is reduced oral bioavailability and an apparent increase in the amount of CYP3A-mediated metabolism (Cummins et al. 2002; Benet 2009). Furthermore, P-gp can enhance the apparent CYP3A4 activity, by acting to reduce the intracellular concentration of the resulting oxidative metabolites which may act as inhibitors of CYP3A (Watkins 1997). Cummins et al. studied the effects of cyclosporine A (a P-gp/CYP3A4 dual inhibitor) and GF120918 (a dual P-gp/BCRP inhibitor) on a dual substrate K77 and felodipine (a specific CYP3A4 substrate) in Caco-2 monolayers model. The extent of metabolism was measured by calculating the extraction ratio across the cells. The apical to basolateral extraction ratio for K77 was decreased by two- and fivefold upon adding GF120918 and cyclosporine A, while the efflux ratio for exclusive CYP3A4 substrate felodipine did not change in the presence of GF120198 but decrease under stimulation by cyclosporine A. The intracellular K77 drug concentration was increased 6- and 4.2-

fold when exposed to P-gp inhibitors but no change with felodipine. In other words, P-gp and CYP3A4 worked in a synergistic manner, and inhibition of P-gp would decrease the intestinal CYP3A4 metabolism (Estudante et al. 2013).

However, Pang (Pang et al. 2009) argued the competition between the enzyme and apical efflux transporter for overlapping substrates that P-gp would limit CYP-mediated metabolism. They doubted with the explanation that P-gp efflux would increase the mean resistance time of drug and is not reliable when taking the biliary secretion and gastrointestinal transit into consideration. What's more, if the hypothesis promoted by Benet that active P-gp can work synergistically with intestinal CYP3A4 to increase the metabolism is valid, then the gut bioavailability of shared substrates should be lower in P-gp-competent mice than in P-gp-deficient mice. However, a study in loperamide, a highly efficient P-gp-mediated efflux and intestinal CYP3A4 metabolism, had shown a controversial result. At low doses (0. 23 mg/kg, 50 μM) which produced intestinal concentration near the apparent K_m for CYP3A4, the gut bioavailability of loperamide was sixfold greater in normal mice than in P-gp-deficient mice, suggesting that P-gp may decrease intestinal metabolism and the effect was large enough to compensate for the increased absorption contributed by P-gp (Dufek et al. 2013b). The result was consistent with in vitro study that the metabolism rate of loperamide was significantly reduced during absorptive flux of loperamide across intestinal tissues from P-gp-competent mouse versus P-gp-deficient mice (Dufek et al. 2013b). At higher doses, the portal plasma concentration and the gut bioavailability were nearly equal in the wild- and mutant-type mice, probably due to the saturation of CYP3A4-mediated metabolism. Thus, the extraction ratio (Cummins et al. 2002; Cummins et al. 2003; Benet et al. 2004) reflected the relative change of produced metabolite over the absorbed parent drug (Dufek et al. 2013b), but the concentration of parent drug would be influenced by P-gp, which couldn't represent the absolute production of the metabolite (Li et al. 2016).

To further understand the role played by P-go in the intestinal first-pass metabolism and gut bioavailability, amprenavir, a drug with moderate efflux by P-gp and high intestinal first-pass metabolism, was also investigated (Dufek et al. 2013a). Interestingly, different from previous reports (Dufek et al. 2013b), portal concentration and gut bioavailability of amprenavir in wild-type mice were nearly identical to those in P-gp-deficient mice even in a sub-saturating concentration. This finding suggested that P-gp had less impact on the intestinal metabolism of amprenavir. The findings were repeated in mice whose CYP450 activity was inhibited by 3-aminobenzotriazole. The results showed that CYP450 attenuated the intestinal absorption by approximately ten-fold irrespective of whether P-gp was functional or not (Dufek et al. 2013a), indicating that CYP450-mediated metabolism dominates intestinal first-pass metabolism of amprenavir. What's more, the consequence of P-gp/CYP3A4 on the intestinal first-pass metabolism would partly rely on whether metabolizing enzymes and transporters are saturated or not. When the local concentrations of the substrate exceed the ability of P-gp, the inhibition of P-gp would have limited influence on the absorption of its substrates. Moreover, if the substrate

concentration was beyond the capacity of CYP3A4 metabolism, inhibition of P-gp may lead to an increased concentration within the enterocytes (Li et al. 2016).

Regional different expression of protein in gut tract should be taken into account because of the heterogeneity of intestinal structure. The expression profiles of P-gp and CYP3A4 were different along the long intestinal tract that the relative level of P-gp increases from the proximal intestine toward the distal region, while CYP3A4 have an opposite distribution patterns (Mouly and Paine 2003a; Thorn et al. 2005; Zimmermann et al. 2005; Watanabe et al. 2013b). Studies using ex vivo rat precision-cut intestinal slices of various regions of intestinal tissues were performed to investigate the P-gp/CYP3A4 interplay of quinidine (dual substrate of P-GP and CYP3A4) under stimulation with different inhibitors (Li et al. 2016). The content of quinidine in slices was enhanced and that of its metabolite, 3-hydroxyquinidine, raised greater when co-administered with the selective P-gp inhibitors CP100356 and PSC833. The dual inhibitors verapamil and ketoconazole increased the quinidine fraction while decreasing the 3-droxylquinidine fraction due to the inhibition of CYP3A4. The content of quinidine in slices was enhanced (1.5- to 2-fold, depending on the region) when co-administered with the selective P-gp inhibitors CP100356 and PSC833. The accumulation of its metabolite, 3-hydroxyquinidine, increased greater by inhibition of P-gp in different regions (approximately 10-fold in duodenum, 20-fold in jejunum, 30-fold in ileum, and <10-fold in colon) corresponds with the P-gp expression along the gut tract, which implied that inhibited P-gp would increase the fraction of metabolized drug.

Considering the species variation and protein abundance of cytochrome enzymes and transporter between human and animals, the same study was then carried out with human precision-cut intestinal slice model (Li et al. 2017a). Applying selective inhibitions of P-gp would significantly affect the intestinal concentration of quinidine with approximately 2.6-fold in the jejunum and 3.8-fold in the ileum but only 1.3-fold in the colon, in line with the low expression of both P-gp and CYP3A4 in the colon. Accordingly, 3-hydroxyquinidine was significantly accumulated in the human intestine slices (approximately 10- to 30-fold, depending on the region, except for 1.2-fold in the colon). The fact that the accumulation rate of metabolite exceeded that of parent drug suggests an improved metabolism by inhibitors. Compared with data in rats (Li et al. 2016), the degree to the increase of quinidine concentration by P-gp inhibition was more remarkable, but the situation to 3-hydroxyquinidine was less in human tissue. These results indicate that the regional differences and species variation should be carefully considered to better represent and mimic a realistic human situation.

As stated above, the consequences of interaction between the metabolizing enzymes and intestinal transporters remains a topic of considerable debate and requires additional preclinical and clinical investigations. Investigations of this sort need to be carefully planned and to take advantage of advances in predictive physiological-based pharmacokinetic models in order to increase the likelihood of generating relevant and mechanistically interpretable results (Darwich et al. 2010; Abuasal et al. 2012; Watanabe et al. 2013a). These considerations include the proper selection of experimental models, (Dufek et al. 2013a, b; Hendrikx et al. 2013),

in vivo intestinal perfusion model (Cummins et al. 2003), or ex vivo precision-cut intestinal slices (Li et al. 2016, 2017a), to allow potential translation to those observed either in simpler in vitro models (cell-based assays) or clinically. For example, compared to the human intestine transport across Caco-2 cell monolayers is only capable of capturing potential interactions with P-gp but not those with the drug metabolizing-enzymes that are expressed in vivo (Li et al. 2017a). Additionally, the experimental conditions that are employed have to be selected in a manner that most closely resembles the in vivo conditions for the specific compound being investigated (Darwich et al. 2010). Although the exact mechanism by which drug transport (primarily efflux) can influence the rate and extent of drug metabolism in the intestine is not fully understood, the substrate specificity between these two important physiological processes in the intestine is likely to exert meaningful effects on the systemic exposure to therapeutics and the potential for clinically-relevant drug-drug interactions (Fig. 8.2).

8.4.2 The Coordination of Apical and Basolateral Intestinal Transport Activities

Drug transporters often have considerable overlap in their substrate specificities with drug metabolizing enzymes. Consequently, the overall disposition of such common substrates can be markedly influenced if the activity of either drug transport or drug metabolism is impaired in the clearance tissues that share the expression of both pathways (Fahrmayr et al. 2012). Epithelial cells lining the gastrointestinal tract require apical and basolateral transporters asymmetrically expressing to function properly (Engevik and Golderning 2018). Like what have been discussed above, there are apical uptake transporters on enterocytes including OCT3, OCTN1/2, PEPT1, and CNT1/2, apical sodium-dependent bile acid transporter (ASBT/SLC10A2), and OATP2B1/1A2 which facilitate the movement of molecules across the intestinal membrane (Klaassen and Aleksunes 2010; Grandvuinet et al. 2012), while the efflux transporters in the apical membrane, like P-gp, BCRP, and MRP2, restrict the movement of respective substrates which under certain conditions may contribute to poor bioavailabilities of drugs (Klaassen and Aleksunes 2010; Grandvuinet et al. 2012). Upon internalization, drug molecules can further interact with additional transporters on the basolateral membrane of enterocyte that catalyze the movement into the blood circulation. These transporters include members of the ABC and SLC transport families such as MRP3, organic solute transporter α(OST-α/*SLC51A)*, organic solute transporter β(OST-β/*SLC51B)*, and MRP4.

The combined activity of apical transporters and basolateral transporters is critical for the efficient vectorial transport across the enterocytes and into the mesenteric circulation, especially for the hydrophilic drug molecules (Muller et al. 2017). The feature of vectorial transport system includes two steps (Ming et al. 2011). Step 1, Apically-located transporters acting as secondary active- or facilitative-transporters mediate the entry of respective substrates into the enterocytes. Step

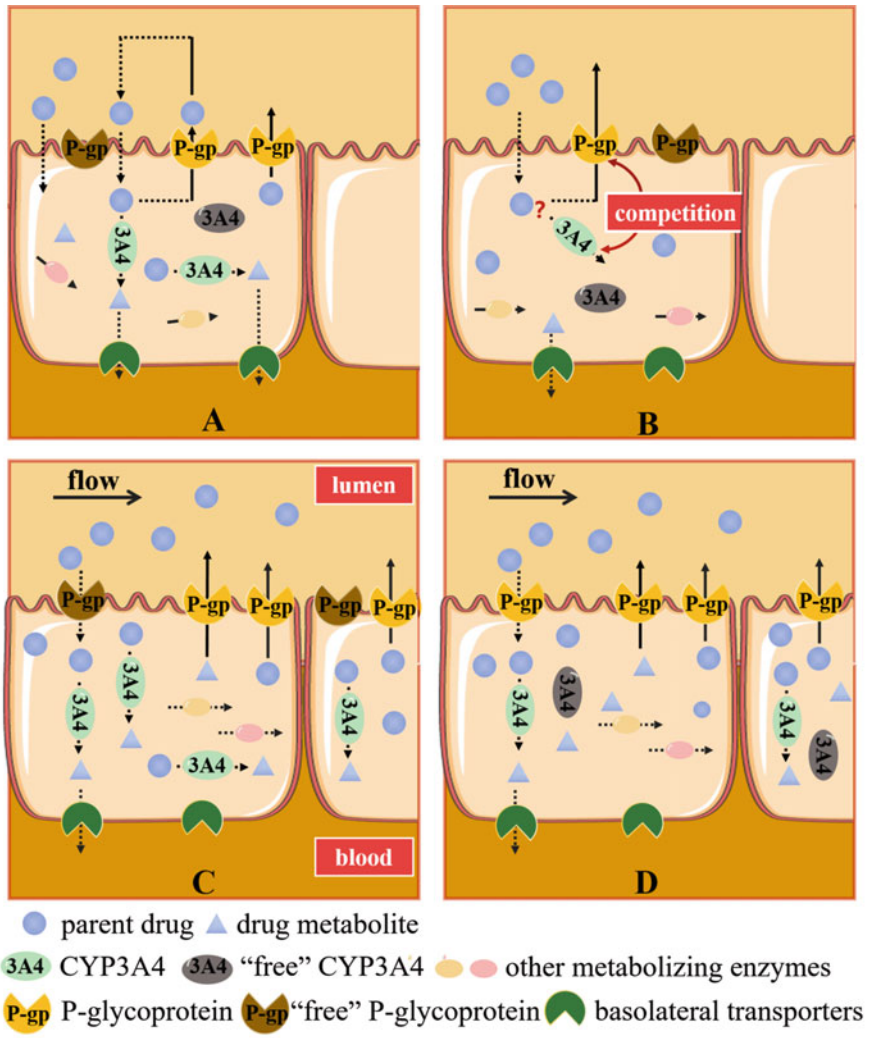

Fig. 8.2 (**a**) Model depicting the "cycling" process of Benet's hypothesis. The drug that absorbed into enterocytes could be metabolized by CYP3A4 or effluxed back into the intestinal lumen. Drug molecules that avoid either pathway have the potential to enter the systemic circulation. Alternatively, dung molecules can either interact directly with CYP3A and be converted into drug metabolites or interact with apical P-gp and be excluded into the intestinal lumen. The drug molecules that are eliminated into the lumen can re-enter into distally-located enterocytes and have an additional chance to either get into the systemic circulation or to interact with CYP3A4. The net effect of repeated efflux along the length of the intestine is the prolongation of tissue residence time and the time that individual molecules have to become susceptible to CYP3A4-mediated metabolism. (**b**–**d**) Other models illustrating the potential interplay between CYP3A4 and P-gp can also be envisaged. These include the possibility for P-gp and CYP3A4 to compete for binding their dual substrates (**b**), under conditions were either P-gp or CYP3A4 activity is subject to saturation (**c**, **d**, respectively)

2, Transporters located at the basolateral membrane accept the internalized molecules and mediate their transport into the blood circulation which eventually coalesce into the portal vein that flows into the liver. Such vectorial transport processes are very common in organs that serve an excretory function such as the liver and kidney. Transporters that facilitate the influx and efflux processes need to function in a collaborative manner in order to enable the efficient elimination of anionic drugs and amphiphilic compounds (Ito et al. 2005). Examples of such processes include the combined activity of hepatic OATPs (OATP1B1, OATP1B3, and OATP2B1) located on the sinusoidal (basolateral) membrane with matched efflux transporters that include MRP2, BCRP and P-gp located at the canalicular (apical) membranes of individual hepatocytes. Additional coupled transport processes include the combined activities of hepatic OCT1 and MATE1 and the sodium taurocholate co-transporting polypeptide (NCTP/*SLC10A1*) and the bile-salt export pump (BSEP/ABCB11). The combined activity of these transporters have been demonstrated to facilitate the hepatic disposition of multiple drugs and their metabolites including several HMG-CoA reductase inhibitors (Ieiri et al. 2009; Rodrigues 2010), telmisartan, ezetimibe glucuronide (Fahrmayr et al. 2012), and morphine glucuronide (Zelcer et al. 2005; van de Wetering et al. 2007). The compounds are either excreted unchanged into the bile or may be metabolized to generate metabolites that are subject to biliary secretion via the various efflux transporters that are expressed on the canalicular membranes of hepatocytes (Grube et al. 2007; Nies et al. 2008; Ieiri et al. 2009; Muller et al. 2017). Admittedly the knowledge regarding the analogous transport process that function to catalyze the vectorial transport across the intestine is not as advanced as that in the liver or kidney. A well-characterized example of such vectorial transport in the intestine is the active absorption of D-glucose via the combined activity of the sodium-dependent glucose transporter (SGLT1) and the facilitative glucose transporter GLUT2/SLC2A2 (Ferraris et al. 2018). SGLT1 facilitates the entry of glucose taking advantage of the sodium ion concentration gradient between the cell's exterior and interior compartments that is generated from the activity of the ATP-dependent Na^+-K^+ exchange pump. Glucose molecules are subsequently transported across the enterocyte basolateral membrane via GLUT2 into the blood circulation. Another well-understood example of vectorial transport in the intestine involves the participation of the apical, sodium-dependent bile acid uptake transporter (ASBT) and basolateral OST-α/β (Dawson 2011). This transport pathway is most active in the distal parts of the small intestine (ileum) and constitutes a key component of the overall entero-hepatic circulation of bile acids in the body. Additional evidence for coupled transport in the intestine (albeit in the secretory direction) comes from in vitro investigation of rosuvastatin (Li et al. 2012) were the measured efflux ratio across colon-derived Caco-2 cells was significantly higher than that across MDCK cells overexpressing human BCRP. This in vitro finding suggested that rosuvastatin flux across biological barriers, such as the intestine, requires the tandem activity of transporters on either side of relevant cells. A similar transport mechanism for fexofenadine is also believed to be functional in the intestine that combines the uptake activity of OATP2B1 on the apical membrane with MRP3 efflux across the basolateral membranes that aids in the intestinal absorption (Ming et al. 2011; Li et al. 2012).

8.4.3 Bile Acid and Gut Transporters

8.4.3.1 The Synthesis of Bile Acids and Enterohepatic Circulation

Bile acid (BA) has been conventionally regarded as the biological detergent responsible for the absorption of lipids, lipid-soluble vitamins, and poorly water-soluble drugs through micellar dispersion (Swann et al. 2011; Enright et al. 2017, 2018). In addition to playing a crucial role in the homeostasis of cholesterol, plentiful studies have shown that bile acids also function as signaling molecules that can modulate lipid (Watanabe et al. 2004; Sun et al. 2017) and glucose (Li and Chiang 2014; Ma et al. 2017) levels and have effects on global metabolic and (Watanabe et al. 2006), inflammatory responses (Ho and Steinman 2016). Bile acids can also play a role in regulating the activity of drug metabolizing enzymes owing to the broad tissue distribution of the bile acid-activated receptors (BARs) including FXR, PXR, CARs, VDR and G protein-coupled bile acid receptors (GPCRs; TGR5) (Swann et al. 2011; Benz-de Bretagne et al. 2014; Li and Chiang 2014) whose activation results in transcriptional activation multiple drug metabolizing enzymes. Impairment of BA synthesis, regulation, or trafficking have all been shown to have profound effects on the pathogenesis of multiple liver diseases including, nonalcoholic fatty liver diseases, diabetes, and overall metabolic function (Fiorucci and Distrutti 2015; Chavez-Talavera et al. 2017; Molinaro et al. 2018).

BAs are synthesized in the liver from cholesterol along a metabolic pathway that involves the participation of at least 17 enzymes (de Aguiar Vallim et al. 2013; Wahlstrom et al. 2016). Briefly, the synthesis proceeds via two pathways as shown in (Fig. 8.3). The classical pathway is responsible for the majority of BA synthesis and is initiated by the highly regulated CYP7A1 enzyme which constitutes the rate-limiting of the pathway. The primary BAs produced in human liver are cholic acid (CA) and chenodeoxycholic acid (CDCA), while rodents produce muricholic acid (MCAs) in addition to those synthesized in humans. BAs undergo conjugation reations resulting in the formation of the respective glycine- or taurine-conjugates (Enright et al. 2018). In the intestine, the conjugated BAs are partially deconjugated and subsequently transformed to the secondary bile acids including lithocholic acid (LCA), MCA, and ursodeoxycholic acid (UDCA) (murideoxycholic acid (MDCA) and ω-MCA in mice) through various intestinal microbiota-mediated reactions (Sayin et al. 2013; Wahlstrom et al. 2016). Approximately 95% of BAs that are secreted into the intestine are re-absorbed into the circulation via ASBT-mediated uptake activity in distal ileum and into the liver via NTCP were individual molecules are enzymatically converted into the conjugated form and recycled into the BA pool (Klaassen and Cui 2015).

The BA transporters and elements in enterohepatic circulation are regulated by a tight feedback control via nuclear receptor FXR in the intestine and liver (Wahlstrom et al. 2016). Activation of FXR in enterocytes mainly by the primary BAs, CDCA and CA results in the downregulation of BA uptake and upregulation of BA efflux mechanisms (Jahnel et al. 2014). Besides, the transcription of FGF15/19 in the

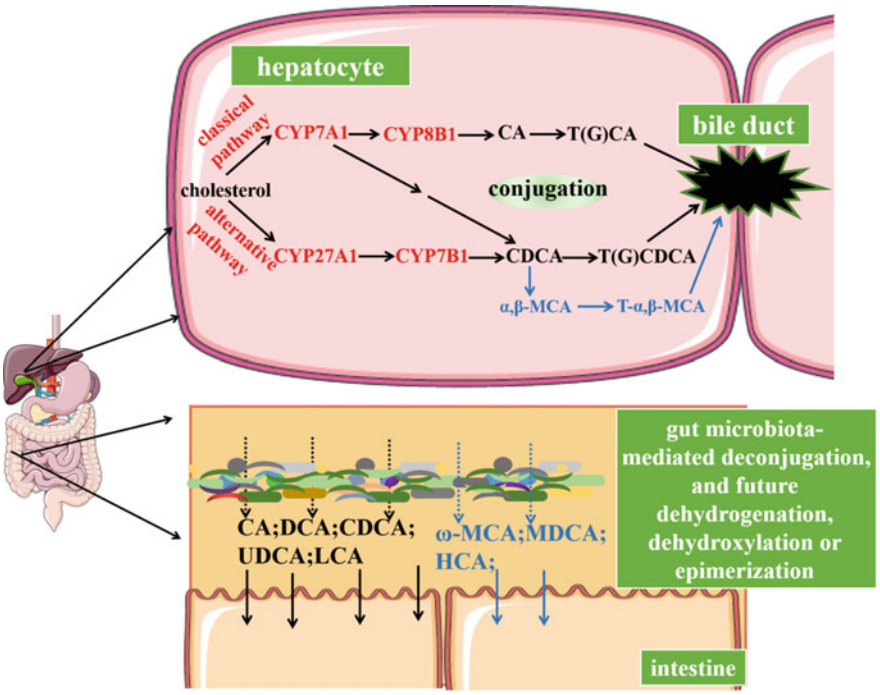

Fig. 8.3 The synthesis and metabolism of bile acids. Bile acids are synthesized in the liver through two pathways: the classical pathway (75%) is mediated by CYP7A1 and CYP8B1 to produce CA and CDCA and the alternative pathway (25%) producing CDCA via CYP27A1 and CYP7B1. In rodents, the majority of CDCA is transformed to α, β-MCA (showed in blue). Most of the primary BA in hepatocyte are conjugated to taurine or glycine prior to their secretion into the bile duct. In the intestine, conjugated BA may undergo deconjugation resulting in the formation of secondary BAs as shown above. At the terminal ileum, the majority (95%) of BAs are reabsorbed and undergo biotransformation reactions in the liver restoring them back to their primary BA state

intestine would be increased, which in turn would be secreted into the portal vein and taken up by hepatocytes. Upon binding to its receptor, FGF15/19 results in the activation the c-Jun N-terminal kinase/extracellular signal-regulated kinase (JNK/ERK) signaling, resulting in the downregulation of CYP7A1 and CYP8B1 to reduce in the rate of BA synthesis (Inagaki et al. 2005; Potthoff et al. 2012).

8.4.3.2 The Intestinal Bile Acid Transporters: ASBT and OST-α/β

In the intestine, BAs are mainly reabsorbed by the brush border via the bile acid transporter ABST in the distal ileum and partly accompanied with the passive absorption in the proximal ileum and colon. The intracellular BAs bind the cytosolic ileal bile acid-binding protein (IBABP; fatty acid-binding protein 6 (FABP6)) and are exported across the basolateral membrane via OST-α/β (Pellicoro and Faber 2007; Klaassen and Aleksunes 2010; Dawson and Karpen 2015), as depicted in

Fig. 8.4. The combined ASBT and OST-α/β activities act as gatekeepers for the intestinal compartment of the enterohepatic circulation of BAs (Ferrebee and Dawson 2015).

ASBT expression in the small intestine is primarily localized to the ileum in rodent animals and human (Dawson 2011) where it catalyzes the reuptake of bile acids that are secreted into the duodenum. ASBT generally possesses a narrow substrate specificity that is somewhat limited to BAs including the major unconjugated BA, CA, DCA, CDCA, and UDCA, as well as their glycine and taurine conjugates (Dawson 2011). Inhibiting or inactivating ABST reduces BA absorption resulting in an increased level of BA in feces. The reduced levels of reabsorbed bile acids activate downstream compensatory mechanisms to induce the *de novo* synthesis and can have the therapeutic effect of reducing systemic cholesterol plasma levels (Lan et al. 2012; Wu et al. 2013; Vivian et al. 2014; Dawson and Karpen 2015).

While the mechanism and modulation of ASBT in the transport of BA in the intestine have been widely recognized, the mechanism for OST-α/β-mediated transport has not been fully elucidated (Dawson 2011). In general, the expression of OST-α is usually accompanied with the expression of OST-β. Interestingly, the

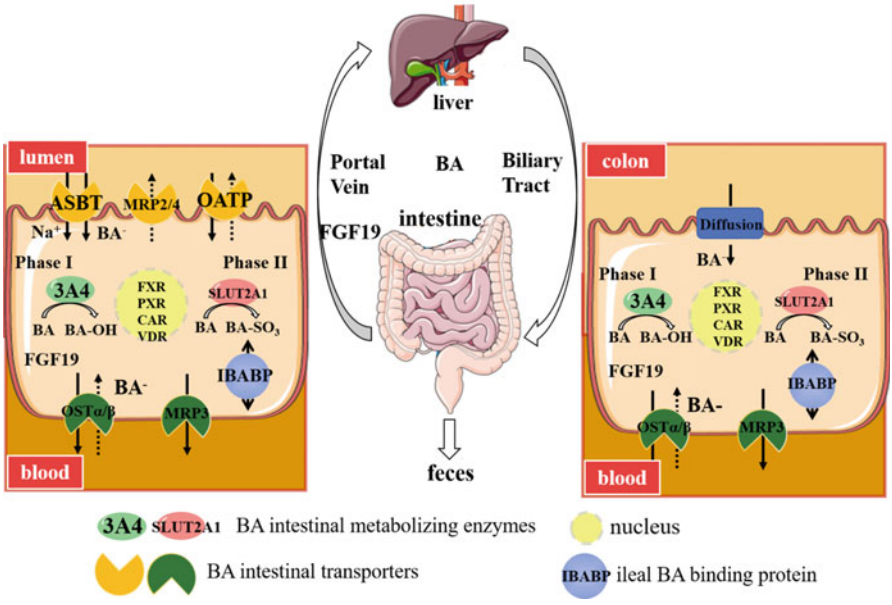

Fig. 8.4 The transport and metabolism of BAs in the intestine via BA transporters, enzymes, and key regulatory nuclear receptors. The apical transporters include ASBT, OATPs (OATP2B1 and OATP1A2), MRP2, and MRP4, and the basolateral transporters contain OST-α/β and MRP3. BA may also be metabolized by cytochrome P450 enzymes (e.g., CYP3A4) and phase II sulfating enzymes (e.g., SULT2A1). Nuclear receptors like FXR, PXR, CAR, and VDR regulate the expression of BA-associated transporters. After stimulation by BA, FGF-19 is synthesized in the small intestine and secreted to the portal vein resulting in the reduction in the *de novo* synthesis of BA in the liver

tissue distribution of OST-α/β parallels that of ASBT, consistent with their role in BA homeostasis (Soroka et al. 2010; Ballatori et al. 2013). Compared with ASBT, OST-α/β have a broader tissue distribution and are able to transport additional substances besides BAs, which include steroids, estrone-3-sulfate, digoxin, and prostaglandin E2 (Fang et al. 2010; Dawson 2011; Ballatori et al. 2013). Interestingly, *Ost-α*$^{-/-}$ mice do not exhibit the classical response to reduced intestinal BA absorption (Rao et al. 2008; Lan et al. 2012), which is partly explained by the induction of compensatory mechanisms that restor BA trafficking. These mechanisms include the increased expression of transporters that are capable of BA efflux including MRP3 (Ballatori et al. 2008, 2013; Lan et al. 2012).

BAs could modulate their own synthesis through the decreased expression of CYP7A1 via FXR-mediated feedback. Moreover, BA transporters expressed in the gastrointestinal tract, namely ASBT and OST-α/β, are also regulated by the direct or indirect impact of FXR activation (Dawson et al. 2009). The influx transporter ASBT could be indirectly downregulated by the enhanced transcription of the repressor short heterodimer partner (SHR) by FXR, while BA could directly interact with FXR elements in the promoter region of OST-α/β, contributing to improved expression of efflux transporter (Soroka et al. 2010). In addition, the FXR-FGF15/19 pathway also contributes to the regulation of the expression of Slc51a and Slc51b (Rao et al. 2008; Ballatori et al. 2013). Some hormones have been proven to induce the expression of ASBT, including the glucocorticoid methylprednisolone and VDR agonists due to their positive activating effect in glucocorticoid receptor (Coon et al. 2010; Claudel et al. 2011) or VDR (Chen et al. 2006; Chow et al. 2010). A set of 30 FDA-approved drugs have been identified to be potent inhibitors of ABST in cell-based assays. These inhibitors include dihydropyridine calcium channel blockers and HMG-CoA reductase inhibitors (Zheng et al. 2009). ion of Multiple chemical substances are believed to act as potential inhibitors of OST-α/β, including spironolactone, sulfobromophthalein, probenecid, and indomethacin (Ballatori et al. 2013). The inhibition or genetic ablation of OST-α/β would be expected to have an effect on the enterohepatic circulation of bile acids in human (Rao et al. 2008; Sultan et al. 2018) however, as mentioned above, such effects are unlikely to have a major effect due to the activation of compensatory BA-efflux pathways.

8.4.4 Gut Microbiota and Bile Acid

It has been estimated that a community of approximately 10^{14} symbiotic microorganisms inhabit the human gastrointestinal tract. The intestinal microbiota is composed of over 2000 bacterial species (Fiorucci and Distrutti 2015; Kim 2018). Various bioactive bacterial compounds may modulate the host metabolism by activating cognate receptors in various cells (Holmes et al. 2012), and one of the most important signaling molecules involved is bile acids (de Aguiar Vallim et al. 2013). The dysbiosis of the intestinal microbiota would alter the bile acid pool significantly (Li et al. 2017c). A recent study showed that four antibiotics including

azithromycin and amoxicillin may significantly increase taurocholic acid (TCA) levels in rat plasma and decrease the level of the unconjugated primary BA and secondary BA (Li et al. 2017b), which suggests the impaired intestinal 7-α-dehydroxylating bacteria (Sayin et al. 2013). Researchers characterized a totally different BA profile in the germ-free and antibiotic-treated rats compared with normal animals via UPLC-MS. In particular, the taurine-conjugated BA experienced a dominant increase in the liver, kidney, and plasma (Swann et al. 2011). The bile acid pool size and composition are inherently linked with microbial community (Staley et al. 2017). The direct mechanism should be attributed to the gut bacterial enzymes which are responsible for transforming the primary acids through deconjugation, dehydrogenation, dehydroxylation, and sulfation reaction into the secondary BA (Staley et al. 2017).

Another mechanism was that the gut microbiota regulates both the BA transporters and metabolism enzymes in a BAR-dependent manner (Sayin et al. 2013; Kuno et al. 2016; Wahlstrom et al. 2016; Jia et al. 2018). The potential vulnerable coding genes include CYP7A1 and CYP8B1 and efflux hepatic transporters BSEP and MDR3 (Swann et al. 2011; Wilson and Nicholson 2017). In germ-free mice, the expression of CYPs was lower than mice exposed with gut microbiota, possibly because LCA increased the expression of the hepatic CYPs via agonizing PXR and CAR (Toda et al. 2009). Besides, the ampicillin-treated mice exhibited a reduced expression level of FGF15 with a concomitant increase in the expression level of ASBT and decreased fecal BA excretion (Miyata et al. 2011). Attenuating the gut microorganism decreased the stimulation toward the transcription factor Gata4, increasing the expression of ileal ASBT, leading to an increase in absorption and a decrease in the synthesis of BA (Beuling et al. 2010; Out et al. 2015).

Different bile acid receptors were disproportionately activated according to the properties of bile acids. The rank-order of FXR and TGR5 activation is CDCA>DCA>LCA>CA and LCA>DCA>CDCA>CA, respectively. In contrast, LCA has been shown to selectively mediate the activation of VDR and PXR (Fiorucci and Distrutti 2015; Song et al. 2015). Consequently and alteration in the BA profiles or the ratio of various BAs act to modulate the expression of downstream metabolizing enzymes and transporters that are involved in the synthesis and transport of BA.

Finally, the role played by enteric bacteria (microbiome) in catalyzing the metabolism of secreted bile acids has been gaining quite a bit of attention and an additional BA regulatory mechanism. Enteric bacteria carry out the biotransformation of secreted bile acids (referred to as primary bile acids) resulting in the formation of secondary bile acids (deconjugation and dehydroxylation). As mentioned previously, these secondary bile acids have varying degrees of activation of various nuclear receptors, that have different downstream regulatory effects on the expression of metabolic and transport pathways (Klaassen and Cui 2015). Moreover, these signaling molecules can have an indirect effect on the expression of the host's drug metabolizing enzymes thereby further influencing the inter-individual response to drug exposures and efficacy (Li and Jia 2013).

8.5 Contribution of Intestinal Drug Transporters to Drug-Induced Toxicity

A large number of transporters are expressed in the intestine and their activity may, under certain circumstances, play a significant role in modulating drug absorption and the potential for downstream toxicities. These transporters when expressed on the membrane of a particular cell can significantly influence the intracellular drug concentration under conditions where their catalytic activity is not saturated. Consequently, the physicochemical properties of drug molecules that are subject to transport would act as the main determinants of the extent by which intestinal transport (uptake or efflux) can influence drug absorption. Highly soluble drugs achieve high concentrations in the intestinal lumen resulting in the saturation of the transport processes. Likewise, the rapid passive movement of highly permeable drugs across cell membranes overcomes the active transport mechanisms in either direction thereby reducing their overall impact on the overall flux down the concentration gradient between the intestinal lumen and the systemic circulation.

8.5.1 Protective Effect of Efflux Transporters Against Toxin-Mediated Damage

P-gp and BCRP in the intestine are mainly expressed on the brush border of intestinal epithelial cells. These efflux transporter acts as a protective mechanism by reducing the absorption of toxic substances that are derived from food sources of therapeutic agents (e.g., the anticancer agents paclitaxel and docetaxel). A clinical trial demonstrated that co-administration of oral P-gp inhibitor cyclosporine A strongly enhanced the oral bioavailability of docetaxel by about tenfold (Malingre et al. 2001a). Similarly, bioavailability of oral paclitaxel in combination with cyclosporine A was eightfold higher than after oral paclitaxel alone (Meerum Terwogt et al. 1999). Co-administration of P-gp inhibitor GF120918 also significantly increased the systemic exposure to oral paclitaxel and in cancer patients (Malingre et al. 2001b). The increases in plasma exposure of paclitaxel and docetaxel would lead to enhancement of their efficacy and/or toxic effects.

8.5.2 Drug Transporters and Enterohepatic Recirculation

For some drugs, enterohepatic recirculation is critical for their ADME process, especially their conjugated metabolites which were subjected to glucuronidation, sulfation, or glycosidation. For example, biliary excretion is the major route for the elimination of saponin from the body (Jin et al. 2015). Due to the large molecular mass (>500 Da), high hydrogen-bonding capacity, and poor membrane permeability

(Liu et al. 2009), the oral bioavailability of saponins is often very low. However, saponins are hydrolyzed to aglycones by intestinal bacteria. Aglycones are easily absorbed by the intestine into circulation. Aglycones in turn undergo phase I and/or phase II metabolism in the liver or intestine resulting in the appearance on the resulting metabolites in the systemic circulation or secretion into the bile that is partly mediated by the activity of efflux transport mechanisms on relevant cell membranes in either tissue. For instance, astragaloside IV is metabolized by intestinal bacteria to brachyoside B and cyclogaleginoside B which are absorbed into circulation and are secreted into the gastrointestinal tract via bile. In gastrointestinal tract, brachyoside B and cyclogaleginoside B could be hydrolyzed again to produce tertiary metabolites (Jin et al. 2015). In summary, the activity of drug transport and gut microbiota work in concert is such situations and the perturbation of either process can result in meaningful effects on therapeutic and/or toxic outcomes (Jin et al. 2015; Chen et al. 2016).

8.5.3 Drug-Induced Enteropathy and Diarrhea

The gastrointestinal toxicity of therapeutic agents often results from their concentrative excretion into the bile with the help of transporters. Some of them is reabsorbed, constructing enterohepatic recirculation. Enterohepatic recirculation may notably affect pharmacokinetic parameters such as increases in plasma half-life, AUC, and bioavailability, finally enhancing therapeutic effects and toxicity of drugs (Malik et al. 2016). In general, drug elimination via bile is mainly mediated by three ABC transporters P-gp, BCRP, and MRP2 (Nies and Keppler 2007; Polgar et al. 2008; Cascorbi 2011).

Typical example is the gastrointestinal toxicity induced by antineoplastic agent irinotecan. In the liver, irinotecan is metabolized to active metabolite SN-38, which is further metabolized to an inactive glucuronic acid conjugate (SN-38G). Both SN-38 SN38-G are mainly excreted into the bile via MRP2/Mrp2 (Chu et al. 1997a, b, 1998). In gastrointestinal tract, SN-38G is hydrolyzed to SN-38 by β-glucuronidase in intestinal microflora (Di Paolo et al. 2006). The mechanism of irinotecan-induced diarrhea has not been fully clarified, but SN-38 accumulation in intestinal tissues is believed to be responsible for its cytotoxicity (Kobayashi et al. 1999; Ikegami et al. 2002). The importance of biliary excretion in irinotecan-induced gastrointestinal toxicity was demonstrated in rat model. In rat, it was found that co-administration of Mrp2 inhibitor probenecid reduced the late-onset diarrhea induced by irinotecan (Horikawa et al. 2002).

Another example is the gastrointestinal toxicity of nonsteroidal anti-inflammatory drugs (NSAIDs). In about 70% of chronic NSAID users, significant small intestinal damage and even bleeding can be observed (Bjarnason et al. 1993; Graham et al. 2005). Recently, one case on indomethacin-induced intestinal injury/bleeding was reported (Mayo et al. 2016). The results demonstrate that bile secretion plays an important role in indomethacin-induced gut injury and appears to support

enterococcal overgrowth of the intestine. The inhibition of cyclooxygenase (COX), the rate-limiting enzyme in synthesis of prostaglandins (PGs) by NSAIDs, is considered to be responsible for NSAID gastrointestinal toxicity (Schoen and Vender 1989), but this could not explain why intestinal damage by NSAIDs still occurs in COX-1 knockout mice (Darling et al. 2004). Moreover, in rat model, it was found that bile flow interruption completely prevented intestinal damage by parenterally administered NSAIDs (Djahanguiri et al. 1973). Moreover, the extent of NSAID-induced intestinal injury has no correlation with the degree of inhibition of PGs synthesis (Ligumsky et al. 1983, 1990; Reuter et al. 1997). These results indicate that additional mechanisms (non-prostaglandin mediated) are also involved in NSAID-induced gastrointestinal injury. The importance of bile salts in the mechanism of NSAID gastrointestinal toxicity has been recognized later. A report demonstrated that NSAIDs exerted their detrimental action on the gastrointestinal mucosa via reducing the protection of phosphatidylcholine against bile salt cytotoxicity (Barrios and Lichtenberger 2000). Compared with wild-type mice, $Fxr^{-/-}$ mice were more prone to develop severe gastric and intestinal injury in response to NSAIDs treating wild-type mice but not $Fxr^{-/-}$ mice with FXR agonists CDCA or GW4064 protected against gastric injury caused by NSAIDs, demonstrating that FXR, a bile acid receptor, was essential to maintain gastric and intestinal mucosal barriers and that NSAIDs induced gastric/intestinal injury via impairing FXR activation by bile acids (Fiorucci et al. 2011).

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Chapter 9
Contributions of Drug Transporters to Blood-Brain Barriers

Li Liu and Xiaodong Liu

Abstract Blood-brain interfaces comprise the cerebral microvessel endothelium forming the blood-brain barrier (BBB) and the epithelium of the choroid plexuses forming the blood-cerebrospinal fluid barrier (BCSFB). Their main functions are to impede free diffusion between brain fluids and blood; to provide transport processes for essential nutrients, ions, and metabolic waste products; and to regulate the homeostasis of central nervous system (CNS), all of which are attributed to absent fenestrations, high expression of tight junction proteins at cell-cell contacts, and expression of multiple transporters, receptors, and enzymes. Existence of BBB is an important reason that systemic drug administration is not suitable for the treatment of CNS diseases. Some diseases, such epilepsy, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and diabetes, alter BBB function via affecting tight junction proteins or altering expression and function of these transporters. This chapter will illustrate function of BBB, expression of transporters, as well as their alterations under disease status.

Keywords Blood-brain barrier · Drug transporter · Alzheimer's disease · Amyotrophic lateral sclerosis · Epilepsy · Parkinson's disease

9.1 General Introduction

Blood-brain interfaces comprise the cerebral microvessel endothelium forming the blood-brain barrier (BBB) and the epithelium of the choroid plexuses forming the blood-cerebrospinal fluid barrier (BCSFB). The BBB and the BCSFB are not only anatomical barriers but also dynamic tissues that express multiple transporters, receptors, and enzymes. Their main functions are to limit free diffusion between

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brain fluids and blood; to provide transport processes for essential nutrients, ions, and metabolic waste products; and to regulate the homeostasis of central nervous system (CNS).

9.1.1 The BBB

BBB, a formidable physical and biochemical barrier, is referred to barrier between blood and the brain or spinal cord parenchyma proper. The function of the BBB is to protect the brain from physiological fluctuations in plasma concentrations of various solutes and from blood-borne substances that can interfere with neurotransmission but at the same time to provide mechanisms for exchange of nutrients, metabolic waste products, signaling molecules, and ions between the blood and the brain interstitial fluid. Brain capillaries are the primary anatomic units of the BBB. The endothelial cells of brain capillaries and the closely apposed pericytes are completely unsheathing by overlapping astrocytic end feet, microglia, and neuronal terminals, constituting a "neurovascular unit" (Redzic 2011) (Fig 9.1).

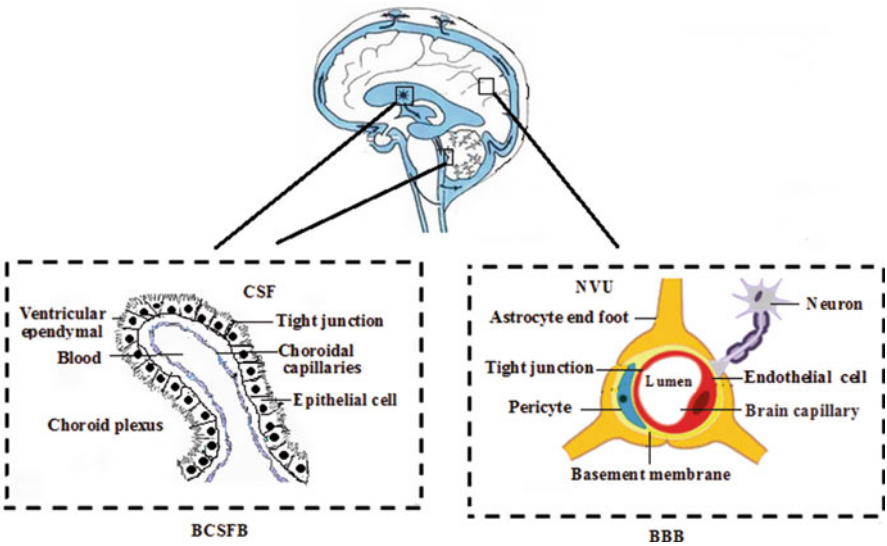

Fig. 9.1 Cellular interfaces between blood, CSF, and brain parenchyma. BBB: the endothelial cells and the closely apposed pericytes are completely unsheathing by overlapping astrocytic end feet and neuronal terminals (NVU). BCSFB: formed by the tight epithelium of the choroid plexuses, which are specialized structures projecting in all four ventricles of the brain and are responsible for the active secretion of CSF. Ependymal cells form the interface between the brain and ventricular CSF. Abbreviations: *BBB* blood-brain barrier, *NVU* neurovascular unit, *CSF* cerebrospinal fluid, *BCSFB* blood-CSF barrier

Different from peripheral vasculature, endothelial cells of brain capillaries are characterized by minimal pinocytotic activity, absent fenestrations, and high expression of tight junction proteins at cell-cell contacts, limiting the exchanges of hydrophilic molecules between the blood and brain (Redzic 2011; Sanchez-Covarrubias et al. 2014). Efflux transporters expressed at the BBB include P-glycoprotein (P-GP), breast cancer resistance protein (BCRP), and multidrug-associated resistance proteins (MRPs), whose functions are to efflux drugs out of CNS (Sanchez-Covarrubias et al. 2014; Strazielle and Ghersi-Egea 2013). On the contrast, influx transporters expressed at the BBB, such as organic anion-transporting polypeptides (OATPs), organic anion transporters (OATs), organic cation transporters (OCTs), and peptide transporters (PEPTs), facilitate drug entry into the CNS (Sanchez-Covarrubias et al. 2014). Most of transporters are transmembrane proteins and located in the luminal or abluminal membranes of the endothelial cells (Fig. 9.2). Thus, the BBB is served as an interface for communication between the CNS and the periphery via different mechanisms including (1) lipid-mediated transport; (2) transporter-mediated transport; (3) receptor- and transporter-mediated transcytosis; (4) absorptive-mediated transcytosis; or (5) non-specific bulk flow transcytosis.

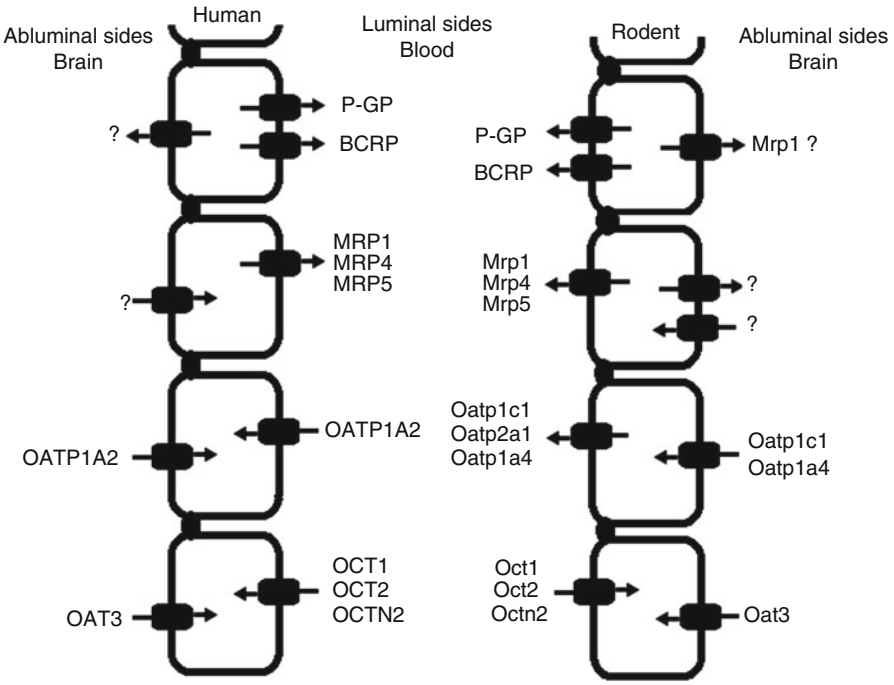

Fig. 9.2 Possible location of some drug transporters in brain microvessel endothelial cells of human and rodents

9.1.1.1 Adherens Junctions and Tight Junctions

Brain endothelial cells and choroid plexus epithelial cells are connected at a junctional complex by the adherens junctions and tight junctions. Adherens junctions consist of multiple proteins including vascular endothelium cadherin, actinin, and catenin (Sanchez-Covarrubias et al. 2014). Unlike other endothelial cells, barrier-forming endothelium and epithelium mainly express cadherin-10 but scarcely show expression of vascular endothelium cadherin. On the contrast, microvessels of glioblastoma multiforme tumors and brain microvessels without BBB properties express only vascular endothelium cadherin not cadherin-10 (Redzic 2011; Williams et al. 2005).

Tight junctions are primarily responsible for restricting paracellular permeability at BBB. Tight junctions consist of multiple proteins including junctional adhesion molecules, occludin, claudins (such as claudin-1, claudin-3, and claudin-5), and zonula occludens proteins (ZOs) (such as ZO-1, ZO-2 and ZO-3) (Sanchez-Covarrubias et al. 2014). Tight junctions form the primary physical barrier component of the BBB. And their function is to restrict paracellular entry of various endogenous and exogenous substances, characterizing high transendothelial electrical resistance (TEER) across the BBB, whose TEER values (1500~2000 Ωcm^2) are considerably higher than those (3~33 Ωcm^2) in other vascular tissues (Redzic 2011; Sanchez-Covarrubias et al. 2014).

9.1.1.2 Astrocytes

Astrocyte end feet ensheathe over 99% of cerebral capillaries, leading to critical cell-cell interactions that directly modulate BBB characteristics. Astrocytes may induce and regulate BBB phenotype via secreting some factors such as transforming growth factor-β (TGF-β), glial cell line-derived neurotrophic factor (GDNF), and basic fibroblast growth factor (BFGF). Additionally, astrocytes increase BBB permeability via Ca^{2+} signaling involving astrocyte-endothelial gap junctions and purinergic transmission. Astrocytes also express various enzymes and transporters such as P-GP, BCRP, and MRPs, contributing to CNS penetration and distribution of drug (Sanchez-Covarrubias et al. 2014).

9.1.1.3 Pericytes

Pericytes are attached at regular intervals to the abluminal side of brain capillary endothelial cells and on the luminal side of the astrocyte end feet. Pericytes are multifunctional cells that contribute not only to vascular contractility and immune responses but also to BBB functional integrity. Several studies have demonstrated that the percentage of vasculature covered by pericytes is positively correlated with "tightness" of the junctions between endothelial cells and inversely correlated with BBB permeability (Sanchez-Covarrubias et al. 2014; Strazielle and Ghersi-Egea

2013). Additionally, pericytes are considered to be essential for ensuring proper localization of endogenous BBB proteins and inducing BBB properties (Al Ahmad et al. 2011; Daneman et al. 2010). All these findings demonstrate roles of pericytes in BBB development and maintenance of its integrity.

9.1.1.4 Neurons

Both brain microvessel endothelial cells and associated astrocyte processes are distinctly connected with noradrenergic, serotonergic, cholinergic, and GABAergic neurons. Loss of direct noradrenergic input from the locus coeruleus was reported to increase BBB susceptibility to effects of acute hypertension, resulting in significantly increased permeability to [^{125}I]-albumin (Ben-Menachem et al. 1982). Similarly, stimulation of the postganglionic parasympathetic fibers of the sphenopalatine ganglion also increased vascular leakage in the brain and increased levels of chemotherapeutic agents (i.e., anti-HER2 monoclonal antibody and etoposide) to therapeutic concentrations in the brain (Yarnitsky et al. 2004).

9.1.1.5 Extracellular Matrix

The extracellular matrix of the basal lamina serves as an anchor for the cerebral microvascular endothelium. In addition, these matrix proteins influence the expression of tight junction proteins and BBB integrity. Disruption of extracellular matrix is associated with loss of barrier function, resulting in increased permeability (Sanchez-Covarrubias et al. 2014).

9.1.2 The BCSFB

The BCSFB, formed by the choroid plexus, is specialized structures projecting in all four ventricles of the brain and referred to barrier between blood and the CSF. Choroid plexuses are villous structures floating in the CSF and attached to the ventricular ependyma by a stalk. The ependyma is continuous with the epithelial layer of the choroid plexuses consisting of a single layer of cells joined together by tight junctions and fenestrated capillaries, forming the BCSFB (Fig. 9.1) and limiting paracellular diffusion of hydrophilic substances. Compared with the BBB, the BCSFB shows lower TEER values, inferring that many solutes more easily permeate the BCSFB than the BBB.

In addition to its barrier function, the main function of choroid plexus is to secrete CSF. CSF secretion is regulated by ion exchange across the epithelium and is driven by activity of Na^+-K^+ ATPase and of carbonic anhydrase. Epithelial cells of choroid plexus also express some transporters including OATs/Oats, PEPTs/Pepts, OCTs/Octs, OATPs/Oatps, MRPs/Mrps, and P-GP.

9.2 Major Drug Transporters in the Brain and Their Functions

Endothelial cells of brain capillaries and choroid plexus highly express ATP-binding cassette (ABC) transporter and solute carrier (SLC) families, constructing BBB components. The ABC transporters expressed at BBB mainly include P-GP, BCRP/Bcrp, and MRPs/Mrps. The SLC transporters expressed at BBB mainly include OATPs/Oatps, OATs/Oats, OCTs/Octs, and PEPTs/Pepts.

9.2.1 P-Glycoprotein (P-GP/ABCB1)

In the brain, P-GP/*ABCB1* is mainly localized to the luminal membranes of brain microvessel endothelial cells and to the apical plasma membrane of choroid plexus epithelial cells. P-GP is also expressed in brain parenchyma cellular compartments such as astrocytes, microglia, and neurons (Sanchez-Covarrubias et al. 2014). Main function of P-GP at the BBB is to protect CNS from exposure to potentially neurotoxins and to maintain the precise homeostatic environment (Abdullahi et al. 2017; Schinkel and Jonker 2012). The important roles of P-GP in CNS protection has been highlighted using *Abcb1*$^{-/-}$ mice (Doran et al. 2005; Schinkel et al. 1994). Compared to wild-type mice, *Abcb1*$^{-/-}$ showed a 100-fold increase in brain uptake of ivermectin, a neurotoxic pesticide, leading to significant increase in ivermectin toxicity (Schinkel et al. 1994). Similarly, *Abcb1*$^{-/-}$ mice showed higher ninefold concentration of asimadoline in the brain and at least eightfold more sensitivity to the sedative effect of asimadoline compared with wild-type mice (Jonker et al. 1999). Direct relationships between increases in brain penetration and absence of the *Abcb1* gene have been demonstrated in other drugs such as amiodarone, loperamide, quinidine, verapamil, and digoxin (Doran et al. 2005; Mayer et al. 1997).

P-GP has an immense substrate profiles including antibiotics, calcium channel blockers, cardiac glycosides, chemotherapeutics, immunosuppressants, antiepileptics, antidepressants, HIV-1 protease inhibitors, opioid analgesic drugs, HMG-CoA reductase inhibitors, and some toxins. P-GP also transports many endogenous substrates including cytokines, lipids, steroid hormones, and peptides (Abdullahi et al. 2017; Schinkel and Jonker 2012). Expression of P-GP at BBB becomes a formidable obstacle to CNS drug delivery, thus limiting the ability to effectively treat CNS disorders.

A variety of attempts have been made to enhance CNS drug delivery and their pharmacological activity on CNS. Several P-GP inhibitors have demonstrated to increase pharmacological activity of P-GP substrates on CNS. For example, antidepressant escitalopram is a P-GP substrate. Coadministration of P-GP inhibitor verapamil might increase levels of escitalopram in the brain of mice and enhance the response to escitalopram in the tail suspension test (O'Brien et al. 2013). Transport of loperamide across BBB is also mediated by P-GP. A report showed that tariquidar

(1 mg/kg) or elacridar (1 mg/kg) significantly increased loperamide levels in the brain of rats by 2.3- and 3.5-fold, respectively. Importantly, the concurrent administration of tariquidar (0.5 mg/kg) and elacridar (0.5 mg/kg) showed stronger inhibitory effect, resulting in the most pronounced opioid-induced clinical signs (Montesinos et al. 2014). P-GP also mediates transport of nimodipine across BBB. It was reported that coadministration of erythromycin or cyclosporine A significantly increased brain exposure of nimodipine (Liu et al. 2003) and enhanced the protection of nimodipine against brain damage in rats and mice (Liu et al. 2002). However, caution must be exercised with the use of pharmacological inhibitors of P-GP for enhancing brain delivery. Specifically, treatment with large P-GP inhibitor doses often results in significant systemic toxicity due to the ubiquitous expression of P-GP throughout the body. Moreover, unfair use of P-GP modulators may weaken effect of P-GP substrates on CNS. For instance, although cyclosporine A increased concentration of escitalopram in the brain of mice and rats (O'Brien et al. 2013, 2014), coadministration of cyclosporine A also exacerbated the severity of behaviors in an escitalopram-induced mouse model of serotonin syndrome, a potentially life-threatening adverse drug reaction associated with serotonergic drugs, and augmented the inhibition of escitalopram serotonin turnover in the prefrontal cortex. Cyclosporine A did not enhance the effect of escitalopram (at effective dose) in the tail suspension test; it might worsen depressive symptom of mice at ineffective dose of escitalopram, inducing 37% increase in the duration of immobility compared with no cyclosporine A (O'Brien et al. 2014). P-GP also mediates transport of phenobarbital across BBB. We once reported that verapamil biphasically altered distribution of phenobarbital in the brain of mice and the pharmacological effect of phenobarbital on CNS. Low doses of verapamil (0.125~0.5 mg/kg) shortened the duration time of phenobarbital-induced loss of the righting reflex, but high doses of verapamil (2~4 mg/kg) prolonged the duration time. In line with this issue, brain concentration of PB was decreased by 0.125 mg/kg verapamil but increased by 2 mg/kg verapamil. In vitro BBB model also showed that the uptake of phenobarbital by rat brain microvessel endothelial cells (rBMECs) was decreased by low concentrations of verapamil (1–25 μM) but increased by high concentrations of verapamil (50–300 μM). The concentration-dependently biphasic regulation was also confirmed in the uptake of rhodamine 123 by rBMECs (Yao et al. 2011). The Al-Shawi's steady-state kinetic model was successfully used to fit concentration-dependently biphasic regulation on P-GP functional activity by verapamil (Yao et al. 2011).

Some diseases alter expression of P-GP at BBB. For example, several evidences have demonstrated that P-GP expression in the brain of Alzheimer's disease (AD) patients is significantly downregulated (Chiu et al. 2015; Vogelgesang et al. 2002; Wijesuriya et al. 2010). Positron emission tomography demonstrated that brain tissues including frontal, parietal, temporal, and occipital cortices and posterior and anterior cingulate of AD patients showed higher uptake of [^{11}C]-verapamil binding potential values, demonstrating decrease in brain P-GP activity (van Assema et al. 2012; Deo et al. 2014). Similarly, downregulation of P-GP has been also demonstrated in the brain of mouse model of AD (Park et al. 2014a, b; Hartz et al. 2010; Qosa et al. 2012). Conversely, animal experiments showed that focal cerebral

ischemia increased P-GP expression at BBB (Cen et al. 2013; Spudich et al. 2006). Overexpression of P-GP also occurred in capillary endothelial cells and astrocytes of patients with medically intractable epilepsy (Loscher and Potschka 2002). Our previous studies demonstrated that the kindling by pentylenetetrazole increased expression and function of P-GP in the brain of rats, leading to low accumulation of phenobarbital (Liu et al. 2007a; Jing et al. 2010), and coadministration of cyclosporine A increased brain phenobarbital concentration and potentiated its antiepileptic effects (Liu et al. 2007a). Some antiepileptic drugs are P-GP substrates. These findings demonstrated contributions of P-GP overexpression at BBB to pharmacoresistance observed in these patients. In addition, some peripheral diseases such as diabetes (Liu et al. 2006, 2007b, 2008a) and liver failure (Fan and Liu 2018) also downregulated P-GP expression and function, enhancing pharmacological effect on CNS.

9.2.2 Breast Cancer Resistance Protein (BCRP/ABCG2)

BCRP/*ABCG2* can actively extrude a broad range of endogenous and exogenous substrates. Within the CNS, BCRP is predominantly expressed at the luminal membrane of microvessel endothelial cells. Moreover, astrocytes and microglia also show BCRP expression (Qosa et al. 2015a; Cooray et al. 2002; Cisternino et al. 2004; Eisenblatter et al. 2003; Sanchez-Covarrubias et al. 2014). The function of BCRP at the BBB is to protect the brain from the toxicity of xenobiotics. Several tyrosine kinase inhibitors, such as gefitinib, imatinib, nilotinib, erlotinib, lapatinib, and sunitinib, are substrates of BCRP; thus the existence of BCRP at the BBB also limits entry of these drugs to their intracerebral targets for treating CNS disorder including brain tumors.

Knockout models of *Abcg2* showed increases in the penetration of therapeutic drugs into the brain such as prazosin (Cisternino et al. 2004), methotrexate (Li et al. 2013a), sorafenib (Agarwal et al. 2011a), elacridar (Sane et al. 2013), and CYT387 (Durmus et al. 2013). The brain distribution of methotrexate, a classic antifolate agent commonly used in chemotherapy of primary CNS lymphoma, is severely limited, and only 5% of the free drug in plasma reaches the brain parenchyma across the BBB (Zhu et al. 2009). Clinical trial showed association of low cerebral penetration of methotrexate with recurrent high-grade gliomas (Blakeley et al. 2009). Animal experiment demonstrated the direct involvement of BCRP in methotrexate transport across the BBB, showing that the entry of the drug to the brain was significantly increased in $Abcg2^{-/-}$ mice compared with the wild-type mice (Li et al. 2013a).

It is noteworthy that in vitro BCRP functional analysis at the BBB often results from corresponding cell lines overexpressing BCRP. However, the in vitro transport activity often may not accurately reflect in vivo BCRP function. For example, both dehydroepiandrosterone sulfate and mitoxantrone are typical substrates of BCRP; *Abcg2* knockout little affected brain uptakes of the two compounds in mice, which

gave a conclusion that Bcrp plays a minor role in the active efflux of the two substrates (Lee et al. 2005a). Imatinib is a substrate of P-GP and BCRP. A report demonstrated that deficiency of *Abcb1* significantly increased brain uptake of imatinib, inducing 5.5-fold increase compared with wild-type mice, but brain uptakes of imatinib in wild-type and *Abcg2*$^{-/-}$ mice were similar (Bihorel et al. 2007). Similarly, in MDCK expressing Bcrp, it was found that cimetidine, alfuzosin, dipyridamole, and LY2228820 are substrates of Bcrp, but *Abcg2* knockout did not affect transport of the three compounds at the mouse BBB (Zhao et al. 2009).

In general, BCRP in the brain is usually co-located with P-GP and also displays substrate overlap with P-GP. Thus BCRP is thought to work with P-GP in BBB for restricting numerous xenobiotics into the brain. Erlotinib, flavopiridol, and mitoxantrone were reported to be substrates of P-GP and BCRP (Kodaira et al. 2010), but only the knockout of *Abcb1: Abcg2* or *Abcb1*, but not *Abcg2*, may increase exposure of the three compounds in the brain of mice (De Vries et al. 2012; Kodaira et al. 2010). An in vitro study demonstrated that Bcrp showed more efficient transport of vemurafenib than P-GP, but *Abcg2* deficiency did not increase brain distribution of vemurafenib in mice. However *Abcb1* deficiency still significantly increased brain distribution of vemurafenib. Importantly, the knockout of both *Abcb1* and *Abcg2* genes resulted in remarkable 80-fold increase in brain distribution of vemurafenib, indicating the "synergistic" role of the two transporters at BBB (Mittapalli et al. 2012). This type of "synergistic" effect of P-GP and BCRP was found with other drugs (Table 9.1) such as CYT387 (Durmus et al. 2013), dasatinib (Chen et al. 2009), elacridar (Sane et al. 2013), erlotinib (De Vries, et al. 2012), gefitinib (Agarwal et al. 2010), rucaparib (Durmus et al. 2015), sorafenib (Agarwal et al. 2011a), and topotecan (de Vries et al. 2007). P-GP and BCRP cooperation implies that the absence of either P-GP or Bcrp alone does not result in an appreciable increase in brain penetration of these dual substrates. The greatest enhancement in brain penetration of dual substrates is always seen when both P-GP and BCRP are absent. In accordance, dual P-GP/BCRP inhibitor elacridar showed the strongest enhancement of brain distribution of gefitinib although P-GP inhibitor (LY335979) or BCRP inhibitor (Ko143) alone little altered brain distribution of gefitinib (Agarwal et al. 2010).

An insight into the mechanism of P-GP and BCRP cooperation can be explained by relative transporter affinities of substrate drugs and relative transporter expression levels at the BBB. LC-MS analysis demonstrated that P-GP protein level in the brain capillary endothelial cells of mice was approximately 3~5-fold higher than those of Bcrp (Agarwal et al. 2012; Kamiie et al. 2008). Significantly higher protein expression levels at the BBB make P-GP appear to be the dominant efflux transporter for many dual substrates, although they have similar affinities to both P-GP and BCRP. Thus BCRP-mediated efflux appears to be minor and becomes apparent only when P-GP or both transporters are absent. That is to say, if a compound has moderate affinity to P-GP, higher P-GP expression levels will compensate for lower transporter affinity, resulting in a pronounced P-GP effect on the efflux of this compound at the BBB. This is true for most dual P-GP and BCRP substrates. The contribution of P-GP to the overall efflux of these drugs across BBB is often larger than that of

Table 9.1 Brain distribution (fold increases in brain/plasma ratios relative to wild-type mice) of dual P-GP and BCRP substrates in knockout mice of *Abcg2* or *Abcb1* or *Abcg2: Abcb1* gene

Drugs	Fold increases			References
	Abcb1$^{-/-}$	*Abcg2*$^{-/-}$	*Abcb1*$^{-/-}$*:Abcg2*$^{-/-}$	
CYT387	2.4	2.5	10.5	Durmus et al. (2013)
Dasatinib	4	1	9	Chen et al. (2009)
Elacridar	3.5	6.6	15	Sane et al. (2013)
Erlotinib	2.9	1.3	8.5	De Vries et al. (2012)
Flavopiridol	1.7	1.3	7.4	Zhou et al. (2009)
Gefitinib	31.1	13.7	108	Agarwal et al. (2010)
Ceritinib	47	0.6	96	Kort et al. (2015a)
Imatinib	4.5	1.0	29.0	Zhou et al. (2009)
Lapatinib	3	1.3	40.0	Polli et al. (2009)
Mitoxantrone	1.7	1.4	8.05	Kodaira et al. (2010)
Prazosin	1.8	1.3	6.2	Zhou et al. (2009)
Regorafenib	1.4	3.7	7.9	Kort et al. (2015a)
Rucaparib	2.3	1.3	9.1	Durmus et al. (2015)
Sorafenib	1.2	3.8	9.7	Agarwal et al. (2011a)
Sunitinib	4.6	1.4	34.4	Oberoi et al. (2013)
Tandutinib	2	1	13	Yang et al. (2010)
Vemurafenib	2.9	0.8	83.3	Mittapalli et al. (2012)
Topotecan	2.0	0.7	3.2	de Vries et al. (2007)

BCRP (Kodaira et al. 2010). Data from MDCKII coexpressing human P-GP and BCRP demonstrated that P-GP and BCRP contributed to similar extents to topotecan transport (Poller et al. 2011). However, *Abcg2* deficiency did not alter topotecan ratio of the brain-to-plasma in mice, although *Abcb1* deficiency still increased brain-to-plasma *AUC* ratios (about 2.0-fold increases) (de Vries et al. 2007). Moreover, BCRP is saturable. For example, sorafenib transport by BCRP was the major determinant at low concentrations, but saturation of BCRP-mediated transport occurred at higher concentrations, where P-GP was still fully active. Similarly, sunitinib was transported equally by P-GP and BCRP at low concentrations, but BCRP-mediated transport became more easily saturated at high concentrations than P-GP-mediated transport (Poller et al. 2011). Another example is CYT387, a JAK1/2 inhibitor. In MDCKII expressing human P-GP or BCRP or mouse Bcrp, it was found that the three transporters mediated CYT387 transport, of which CYT387 was more efficiently transported by mouse Bcrp. Mouse experiment showed that at 2 h following dose, CYT387 accumulations in brains of *Abcg2*$^{-/-}$and *Abcb1*$^{-/-}$ mice, were increased to 2.5-fold and 2.4-fold of wild mice, respectively, indicating that both Bcrp and P-GP are equally important in restricting brain entry of CYT387 at 2 h. Interestingly, at 8 h following dose, the role of Bcrp in brain transport of CYT387 across BBB appeared to be more prominent than that of P-GP. At 8 h, brain accumulations of CYT387 were increased to 5.6-fold in *Abcg2*$^{-/-}$ and 3.6-fold in *Abcb1*$^{-/-}$ compared to wild-type mice, respectively, inferring that Bcrp was

saturated at high concentrations of CYT387 (Durmus et al. 2013). On the other hand, some compounds, such as sorafenib, regorafenib, and dantrolene, have a significantly higher affinity for BCRP than for P-GP (Agarwal et al. 2011a, b; Kort et al. 2015b; Kodaira et al. 2010). Thus, Bcrp is the dominant transporter in keeping these drugs out of the brain, and an effect of P-GP on drug penetration is only noticeable in *Abcg2*$^{-/-}$ and *Abcb1*$^{-/-}$:*Abcg2*$^{-/-}$ mice.

Interestingly, expression of P-GP in the mouse BBB was reported to be higher than that of Bcrp (Agarwal et al. 2012; Kamiie et al. 2008), but mRNA levels of BCRP in human brain capillaries were about 8-fold higher (Dauchy et al. 2008) or 1.6-fold (Shawahna et al. 2011) than P-GP mRNA. Moreover, Abcb1 deficiency induced threefold increase of Bcrp mRNA in the brain capillaries compared to wild-type mice (Cisternino et al. 2004) without altering expression of Bcrp protein (Agarwal et al. 2012). Thus, whether BCRP may compensate for the loss of P-GP at the BBB should be further investigated. No evidences showed effect of *Abcg2* deficiency on expression of P-GP at BBB (Agarwal et al. 2012).

9.2.3 *Multidrug Resistance-Associated Proteins (MRPs/ABCCs)*

The primary role of MRPs is to extrude xenobiotics from cells, thereby contributing to MDR development. MRPs differ from P-GP in that their substrate profile is more restrictive. MRPs generally transport organic anions and their glucuronidated, sulfated, and glutathione-conjugated metabolites. However, the expression and distribution of MRPs at the BBB and in the choroid plexus often show controversial findings due to discrepancies in mRNA and protein expression, species specificity, or technical differences. For example, MRP1, MRP2, MRP3, MRP4, and MRP5 mRNA were detected in brain samples of human, among which expression of MRP5 mRNA showed the highest expression, but MRP1, MRP2, MRP3, and MRP4 mRNA showed considerable variation, and MRP6 mRNA was below detectability. Immunofluorescence analysis showed that MRP1, MRP4, and MRP5 were primarily localized in the luminal membrane of capillary endothelial cells, but no reactivity for the MRP2 or MRP3 proteins (Nies et al. 2004). Human brain microvascular endothelial cells (HBMECs) exhibited the strongest expression of MRP1 mRNA, followed by MRP4, MRP5, and MRP6, but MRP2 and MRP3 showed very weak expression (Kubota et al. 2006). Expressions of MRP1, MRP5, and MRP6 mRNA were induced by epilepsy to 2.5-, 1.3-, and 3.3-fold of the normal brain cortex, respectively (Kubota et al. 2006). Distributions of MRP1 and MRP2 are heterogeneous within the resected region. MRP1 is distributed mainly on parenchymal cells in the parahippocampal gyrus, but not in the hippocampus. By contrast, in one case out of five patients, it was found that MRP2 was present in endothelium rather than parenchymal cells in the hippocampus (Kubota et al. 2006).

Data from comparison of five species (human, rat, mouse, pig, and cow) showed that mRNA levels of MRPs/Mrps were mainly enriched in brain microvessel endothelial cells. MRP1/Mrp1 was detected at high levels in human, rat, and cow, while expression was lower in mouse and negligible in pig. Conversely, MRP3/Mrp3 expression in mouse and pig was higher than other species. The highest expression of MRP4/Mrp4 was found in rodents, followed by pig and human. MRP5/Mrp5 was highly expressed in brain endothelial cells from human, rat, pig, and cow, with lower expression in mice. Rats showed substantial expression of Mrp6 compared to other species. No significant expression of MRP2/Mrp2 was detected in the brain of indicated species (Warren et al. 2009).

Mrp2 expression in the brain of mice is dependent on strain. Different from C57BL/6, Swiss, and SVJ mice, FVB mice lack Mrp2 in the brain vascular and choroid plexus endothelium although Mrp2 is still present in the liver and kidney of the strain, indicating that FVB mice represent a spontaneous, brain-specific "knockout" of Mrp2. In C57BL/6 mice, Mrp1, Mrp2, and Mrp5 are present at specific subcellular localizations in the BBB, where Mrp5 is located in the luminal membrane of endothelial cells, whereas Mrp2 is present in both the abluminal and luminal membrane (and possibly intracellularly), and Mrp1 predominates in the abluminal membrane of capillary endothelial cells. Mrp1, Mrp2, and Mrp3 are also located in basolateral of the membrane of epithelial cells in choroid plexus. Importantly, selective localization of Mrp3 is in gap junctions of choroid plexus (Soontornmalai et al. 2006).

In rat, Mrp1 is primarily localized to abluminal membrane of microvessels and choroid plexus epithelial cells. Mrp4 is predominantly localized to luminal membrane of the microvessel and the lateral surface of choroid plexus epithelial cells. Mrp5 mRNA was detected in rat microvessels at levels comparable to Mrp4 (Roberts et al. 2008; Warren et al. 2009). Mrp5 protein, very weak, is apparent in both non-endothelial cells and microvessels throughout the brain, as well as throughout choroid plexus epithelial cells (Roberts et al. 2008). MRP4/Mrp4 was reported to be located to luminal membrane of rat and mouse brain capillaries or basolateral membrane of human and murine choroid plexus (Leggas et al. 2004; Nies et al. 2004; Roberts et al. 2008), an almost equal distribution of MRP4 protein was detected in the apical and basolateral plasma membrane of bovine capillary endothelial cells (Zhang et al. 2004).

Roles of Mrp1 at BBB have been confirmed by animal experiments. It was reported that the downregulation of Mrp1 in the choroid plexus of rats using antisense targeting Mrp1 significantly lowered the efflux of opioids (such as morphine, oxymorphone, deltorphin II, and [D-Pen2, D-Pen5]encephalin) from the brain into the periphery. Dose-response study revealed that downregulation of Mrp1 significantly enhanced potency of systemic morphine (ED_{50}: 3.5 mg/kg for saline treatment and 1.3 mg/kg for antisense treatment) and oxymorphone (ED_{50}: 0.2 mg/kg for saline treatment and 0.08 mg/kg for antisense treatment). Downregulating Mrp1 also significantly lowered the analgesic response of supraspinal morphine and oxymorphone, shifting the dose-response curve approximately 5~6-folds to the right, which was attributed to the diminished secretion of the drug into the systemic circulation. The findings in rats were further confirmed in *Abcc1*$^{-/-}$ mice (Su and Pasternak 2013).

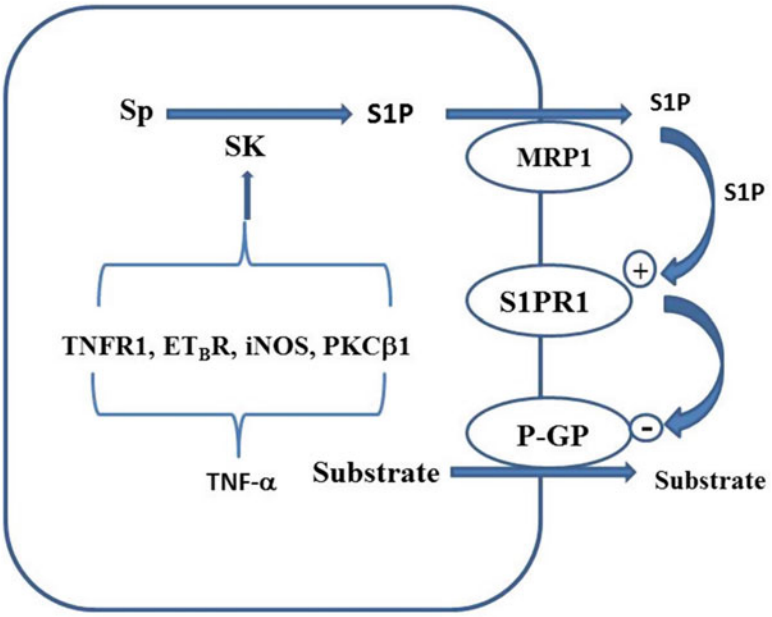

Fig. 9.3 MRP1 reduces P-GP basal P-glycoprotein activity in the BBB via sphingolipid signaling. Sphingosine kinase (SK) phosphorylates sphingosine (Sp) to sphingosine-1-phosphate (S1P). S1P was effluxed out of cell via Mrp1 and then activates sphingosine-1-phosphate receptor (S1PR1), leading to inhibition of P-GP function. TNF-α regulates SK activity via tumor necrosis factor receptor 1 (TNFR1), endothelin receptor B (ET_BR), iNOS, and PKCβ1 signaling

MRP1 also affects function of P-GP at BBB via sphingolipid signaling (Fig. 9.3). Sphingosine kinase phosphorylates sphingosine to sphingosine-1-phosphate intracellularly, and then sphingosine-1-phosphate is transported out of the cells by MRP1/Mrp1 (Mitra et al. 2006) and reduces P-GP function (Cannon et al. 2012; Cartwright et al. 2013) via activating sphingosine-1-phosphate receptor 1(S1PR1). For example, tumor necrosis factor alpha (TNF-α), sphingosine, sphingosine-1-phosphate, S1PR agonist fingolimod (FTY720), and its active, phosphorylated metabolite, FTY720P, reduced P-GP transport activity in the brain and spinal cord capillaries isolated from wild-type mice. These reductions were abolished by a specific S1PR1 antagonist. In line with this issue, neither TNF-α nor sphingosine reduced P-GP activity in the brain and spinal cord capillaries isolated of *Abcc1*$^{-/-}$ mice, but sphingosine-1-phosphate and FTY720P still showed inhibitory effect on P-GP function (Cartwright et al. 2013). Nonselective Mrp inhibitor MK571 abolished the reduction of P-GP function by sphingosine but not sphingosine-1-phosphate in rat brain capillaries (Cannon et al. 2012). MRP1/Mrp1 also mediates transport of other signal molecules such as cAMP, cGMP, leukotriene C4, and certain eicosanoids (prostaglandins), some of which are involved in inflammatory response (Cole 2014), indicating that MRP1 may affect P-GP function via releasing inflammatory molecules.

Some antiepileptic drugs such as phenytoin are MRP2 substrate. Overexpression of MRP2 was also found in epileptogenic brain tissue of patients with pharmacoresistant epilepsy (Dombrowski et al. 2001). *Abcc2*-deficient rats showed significantly higher extracellular brain levels of phenytoin compared with the normal background rats. MRP inhibitor probenecid significantly increased extracellular brain levels of phenytoin. In the kindling model of epilepsy, coadministration of probenecid significantly increased the anticonvulsant activity of phenytoin. In kindled *Abcc2*-deficient rats, phenytoin exerted a markedly higher anticonvulsant activity than in normal rats (Potschka et al. 2003). These results indicate contributions of MRP2 overexpression at BBB to pharmacoresistant epilepsy. Interestingly, the *Abcc2*-deficient rats exhibited a significant upregulation of P-GP expression and function in brain capillary endothelial cells, compensating for the lack of Mrp2 at BBB (Hoffmann and Löscher 2007).

Mrp4 function in the brain has been demonstrated using *Abcc4*$^{-/-}$ mice. It was found that topotecan concentration in the brain of *Abcc4*$^{-/-}$ mice at 6 h following intravenous dose (2 mg/kg) was six times higher compared with wild-type mice. Similarly, topotecan concentration in CSF of *Abcc4*$^{-/-}$ mice was almost tenfold than that of wild-type mice at each tested time point based on CSF samples via microdialysis (Leggas et al. 2004). Ro 64-0802, a pharmacologically active form of the anti-influenza virus drug oseltamivir, is a MRP4 substrate. *Abcc4*$^{-/-}$ mice also showed four- to sixfold greater brain/plasma concentration ratio of Ro 64-0802 than wild-type mice following receiving either oseltamivir or Ro 64-0802 (Ose et al. 2009). Moreover, brain/plasma concentration ratios of 9′-(2′-phosphonylmethoxyethyl)-adenine, methotrexate, raltitrexed, and cyclophosphamide in *Abcc4*$^{-/-}$ mice were significantly higher than those in wild-type mice (Belinsky et al. 2007; Kanamitsu et al. 2017). Prostaglandin E2 (PGE2) acts as a modulator of synaptic signaling and excitability in the brain, whose concentrations of PGE2 are maintained at appropriate levels for normal brain function by regulatory systems. However, PGE2 is barely inactivated enzymatically in adult brain; efflux transport systems in BBB and BCSFB are the primary cerebral clearance pathways for PGE2 in the brain. Efflux of PGE2 across BBB is also mediated by MRP4. MRP4-expressing membrane vesicles showed significant uptake of [^{3}H]PGE2, which was significantly inhibited by cefmetazole, cefazolin, cefotaxime, ceftriaxone, and ketoprofen. Intravenous dose of cephalosporins might significantly inhibit [^{3}H]PGE2 efflux from the brain across BBB of mice (Akanuma et al. 2010). PGE2, an important signal molecule, plays key roles in multiple brain pathophysiological processes including modulation of synaptic plasticity and neuroinflammation. In mouse model, it was found that inflammation significantly reduced PGE2 efflux from the brain (Khuth et al. 2005). Lipopolysaccharide (LPS)-induced inflammation also markedly decreased elimination of [^{3}H]PGE2 from the brain, although the decrease was partly attributed to the decreased expression of Oat3 and Oatp1a4. In addition, either intracerebral or intravenous pre-administration of cefmetazole but not cefazolin further enhanced inhibition of PGE2 elimination from the brain in LPS-treated mice (Akanuma et al. 2011). These findings indicate that in order to avoid unexpected adverse CNS effects, it may be important in the treatment of inflammatory and infectious diseases to use drugs without affecting clearance of PGE2 at the BBB.

9.2.4 *Organic Anion Transporter Polypeptides (OATPs/SLCOs)*

OATP/Oatp expression has been identified in BBB endothelial cells and choroid plexus epithelial cells. OATPs/Oatps localized to CNS barriers include Oatp1a1, Oatp1a4, Oatp1a5, Oatp1c1, and Oatp2a1 for rodents and OATP1A2, OATP1C1, and OATP2B1 for humans. In BBB and BCSFB, OATPs/Oatps are responsible for CNS uptake of a vast array of amphipathic, organic compounds. OATPs/Oatps have also been detected in brain parenchyma cellular compartments such as astrocytes and neurons (Sanchez-Covarrubias et al. 2014).

9.2.4.1 Human OATPs at BBB and BCSFB

Although expression of Oatps at rodent BBB has been well established, identification of OATPs at the human BBB has been often controversial. For example, immunofluorescent staining demonstrated expression of OATP1A2 at frontal brain cortex of human (Lee et al. 2005b), but a proteomic study demonstrated that all OATPs including OATP1A2 were below the detection limit of the approach (Uchida et al. 2011). It should be noted that the brain tissue samples in the study came from subjects who died of peripheral diseases (Uchida et al. 2011). These diseases may modulate expression of BBB transport proteins. Therefore, the proteomic data could not demonstrate the absence of OATPs at BBB of healthy humans.

It is widely accepted that OATP1A2 is expressed at human BBB, which is localized to both the luminal and abluminal membranes of human capillary endothelial cells (Lee et al. 2005b). Well-known substrates of OATP1A2 include therapeutic agents (such as antibiotics, antihistamines, antineoplastic drugs, beta-blockers, cardiac glycosides, endothelin-1 receptor antagonists, HIV-1 protease inhibitors, HMG-CoA reductase inhibitors, neuromuscular blocking agents, opioid analgesic peptides, and bromosulfophthalein) and endogenous compounds (such as bilirubin, estradiol-17β-glucuronide, estrone-3-sulfate, glycocholate, PGE2, taurocholate, triiodothyronine, and tetraiodothyronine). Two different OATP3A1 splice variants (OATP3A1-v1 and OATP3A1-v2) were identified at BCSFB of human with differing localization. OATP3A1-v2 lacks 18 amino acids (aa) at the COOH-terminal end (692 aa) but is otherwise similar in sequence to OATP3A1-v1 (710 aa). OATP3A1-v1 and OATP3A1-v2 are located at the basolateral membrane and the apical membrane of the BCSFB, respectively. Both variants are able to transport drugs (i.e., cyclic oligopeptides BQ-123) and physiological substrates (i.e., PGE1, PGE2, tetraiodothyronine, and vasopressin) (Roth et al. 2012; Huber et al. 2007).

Other OATP isoforms such as OATP1C1 and OATP2B1 have been also detected in human brain tissue, but the exact localization and functional expression of these isoforms at CNS barriers have yet to be determined. Moreover, these OATPs are also expressed in glial cells, suggesting a potential role for OATPs as determinants of CNS drug distribution. OATP1C1 has a more restrictive substrate profile compared

to other OATP1 family members. OATP1C1 primarily mediates transport of thyroid hormones across BBB. But, OATP2B1 mediates transport of many therapeutic compounds (such as aliskiren, atorvastatin, benzylpenicillin, ezetimibe, bosentan, fexofenadine, fluvastatin, glibenclamide, pravastatin, and rosuvastatin) and endogenous compounds (such as dehydroepiandrosterone-3-sulfate, pregnenolone sulfate, and estrone-3-sulfate).

9.2.4.2 Rodent Oatps at BBB and BCSFB

Oatp1a1, a 670 amino acid protein, is expressed at the choroid plexus. Oatp1a1 substrates include bile salts, organic anions, organic cations, and drugs (such as pravastatin and fexofenadine), but functional relevance of Oatp1a1 at the choroid plexus has been not identified. Oatp1a4, the rodent orthologue of human OATP1A2, is expressed at the luminal and abluminal membranes of the rodent BBB and at the choroid plexus. Its substrates include therapeutic drugs (such as opioid analgesic peptides, HMG-CoA reductase inhibitors) and endogenous organic cations (such as bile salts, hormones, and peptides). Along with Oatp1a5 at BCSFB and Oatp1c1 at BBB, Oatp1a4 is responsible for thyroid hormone uptake into the CNS. Oatp1a4 is also the primary drug-transporting Oatp isoform expressed at the rat BBB. For example, Oatp1a4 mediates blood-to-brain transport of [D-Pen2, D-Pen5]-enkephalin and pravastatin. Moreover, pathological stressors such as peripheral inflammatory pain (Ronaldson et al. 2011) and hypoxia/reoxygenation (Thompson et al. 2014) may increase expression and function of brain Oatp1a4 expression via downregulating TGF-β/ALK5 signaling pathway. In the presence of P-GP inhibitor, the relative contribution of Oatp1a4 to brain uptake of [D-Pen2, D-Pen5]-enkephalin was increased from 56% in saline controls to 71% in animals subjected to peripheral inflammatory pain (Ronaldson et al. 2011). These data indicate that the transporter may be a useful target for delivery of peptide therapeutics to the brain.

Oatp1a5, the rodent orthologue of human OATP1A2, is the most highly expressed Oatp family member at the rodent BCSFB. Specifically, Oatp1a5 is localized to the apical brush border membrane of choroid plexus epithelial cells. Substrates of Oatp1a5 include bile salts, steroid conjugates, and thyroid hormones. Its presence on the opposite membrane as Oatp1a4 and Oatp1c1 can facilitate movement of its substrates into or out of the CSF.

Oatp1c1, a 716 amino acid protein, is expressed at both BBB and BCSFB. At the BBB, Oatp1c1 has been localized to the luminal and abluminal membranes of rodent BBB endothelial cells. In the choroid plexus, Oatp1c1 is primarily expressed at the basolateral membrane of choroid plexus epithelial cells. Oatp1c1 has a more restrictive substrate profile than most other OATPs/Oatps, functioning primarily as a high-affinity thyroxine transporter. In rodents, Oatp1c1 along with monocarboxylate transporter 8(MCT8) mediates uptake of triiodothyronine and thyroxine into the brain. Oatp1c1 deficiency was demonstrated to significantly decrease uptake of triiodothyronine and thyroxin by 50% of wild-type mice. Importantly, double knockdown of Mct8 and Oatp1c1 strongly reduced uptake of both triiodothyronine

and thyroxine into the brains compared with single-mutant or wild-type mice, leading to impairment of brain development and function (Mayerl et al. 2014).

Oatp2a1, a PGE2 transporter, is mainly expressed in astrocytes, pericytes, and choroid epithelial cells and cerebral endothelial cells of rats. Oatp2a1 is mainly located to luminal plasma membrane of cerebral endothelial cells. LPS treatment did not affect expression of Oatp2a1 but altered localization pattern of Oatp2a1, shifting from predominantly luminal to throughout the cytoplasm (Kis et al. 2006). The results indicate that the alteration in Oatp2a1 characteristic affect transport of prostaglandins across BBB and BCSFB, leading to prostaglandin dyshomeostasis in the brain and initiating fever response.

9.2.5 Organic Anion Transporters (OATs)

OATs/Oats are also expressed at the choroid plexus and at brain microvasculature. Among OATs/Oats, OAT3/Oat3 is the most highly expressed OAT/Oat isoform in the brain. Substrates of OAT3/Oat3 include para-aminohippuric acid, estrone sulfate, taurocholate, ochratoxin, benzylpenicillin, cimetidine, and ranitidine. OAT3/Oat3 plays a critical role in the transport of anionic metabolites of neurotransmitters (such as epinephrine, norepinephrine, dopamine, and serotonin). OAT3/Oat3 is localized to the apical membrane of choroid plexus epithelial cells where it mediates entry of substrates into the choroid plexus and out of the CSF. In rat, Oat3 is also localized to both the basolateral membrane and the apical membrane of brain capillary endothelial cells, which allows for both blood-to-brain and brain-to-blood transport of its substrate drugs.

9.2.6 Organic Cation Transporters (OCTs)

Expressions of OCTs/Octs in the brain are regional. In rat brain, both Oct2 and Oct3 mRNAs are expressed predominantly in regions located at the brain-cerebrospinal fluid border, with Oct3 mRNA expression extending to regions that belong to monoaminergic pathways such as raphe nuclei, striatum, and thalamus (Amphoux et al. 2006). Both Oct1 and Oct2 proteins are mainly expressed at the luminal side of brain microvessel endothelial cells and adult rat brain endothelial cells, where they mediate transport of toxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Lin et al. 2010). Importantly, both the mRNA and protein levels of Oct1 and Oct2 in the brain of aged mice are lower than younger mice. In accordance with this issue, the aged mice and *Oct1/2*$^{-/-}$ mice showed much lower brain extracellular levels of MPTP and its metabolite 1-methyl-4-phenyl-pyridinium (MPP^+). MPTP-induced neurotoxicity in the aged mice and *Oct1/2*$^{-/-}$ mice was also less than that in younger mice and wild-type control mice. On the contrast, intrastriatal infusion of low-dose MPTP showed more severe dopaminergic toxicity in both aged mice and *Oct1/2*$^{-/-}$

mice (Wu et al. 2015), which was attributed to lower efflux of MPTP. Both Oct2 and Oct3 are also detected in the main aminergic projection regions, such as the cortex, hippocampus, thalamus, hypothalamus, amygdala, and hindbrain, as well as in most aminergic nuclei, raphe, locus coeruleus, and tuberomammillary nucleus, where they mediate the transport of neurotransmitters, such as serotonin, dopamine, and norepinephrine, and affect vulnerability to mood-related disorders such as depression or anxiety and to neurodegenerative diseases such as Parkinson's and Alzheimer's diseases (Couroussé and Gautron 2015). Oct3 is also expressed in glial and ependymal cells in the dorsomedial hypothalamus, which is considered to be a stress-sensitive component of the mechanisms regulating monoamine clearance within the dorsomedial hypothalamus. The Oct3-mediated transports of monoamines in dorsomedial hypothalamus were to be inhibited by corticosterone, indicating that corticosterone regulation of Oct3 may contribute to acute regulation of integrated behavioral and physiological responses to stress (Gasser et al. 2006, 2009). Moreover, brain Octs also transport therapeutic drugs/toxins (such as amphetamines, oxycodone, and MPTP) at the BBB, in turn, affecting their CNS activity/toxicity (Couroussé and Gautron 2015).

Octn1 has been detected in rodent's spinal cord, choroid plexus, hippocampus, cortex, and cerebellum. Expression of OCTN1 in human brain tissue needed further confirmation. In addition to carnitine, OCTN1/Octn1 transports quinidine, choline, nicotine, cimetidine, and clonidine. OCTN2/Octn2 mRNA expressed neurons from the hippocampus, cerebellum, and cerebral cortex. OCTN2/Octn2 protein expression has been detected in luminal membrane of primarily cultured brain capillary endothelial cells from cows, pigs, rats, and humans, whose function is to transport acetyl-L-carnitine from the blood to the brain (Kido et al. 2001; Inano et al. 2003).

9.2.7 Peptide Transporter 2 (PEPT2)

PEPT2/Pept2 has been detected in the cerebral cortex, cerebellum, choroid plexus, and astrocytes. PEPT2/Pept2 has a high affinity for a di- and tripeptides such as glycyl-L-glutamine (Gly-Gln). Pept2 is localized to the apical membrane of the choroid plexus epithelial cells, where it mediates efflux of neuropeptides, peptides, peptidomimetics, and peptide-like drugs from CSF into the blood. For example, CSF-to-plasma concentration ratio of carnosine in *Pept2*$^{-/-}$ mice was reported to be eightfold greater than that in wild-type mice following intravenous administration. In accordance, concentrations of carnosine in choroid plexus of *Pept2*$^{-/-}$ mice were significantly lower than that in wild-type mice (Kamal et al. 2009). *Pept2* deficiency may increase CNS response or neurotoxicity of some drugs. 5-aminolevulinic acid, a heme precursor, is a Pept2 substrate. In *Pept2*$^{-/-}$ mice, administration of 5-aminolevulinic acid was reported to reduce survivability and worsen neuromuscular dysfunction, accompanied by 8–30-fold higher concentrations of 5-aminolevulinic acid in CSF compared to wild-type mice (Hu et al. 2007). Similarly, L-kyotorphin, an endogenous analgesic neuropeptide, is also a Pept2 substrate.

It was found that clearance of L-kyotorphin from CSF of *Pept2*$^{-/-}$ mice was also slower than that in wild-type mice. Dose-response analysis showed that the ED_{50} of L-kyotorphin in *Pept2*$^{-/-}$ mice was only one-fifth in wild-type mice following intracerebroventricular administration (Jiang et al. 2009). Efflux of cephalosporins such as *cefadroxil* in the brain is mediated by Pept2 (Smith et al. 2011; Chen et al. 2014, 2017). In fact, cefadroxil is also a substrate of other transporters including OATs and MRPs (Chen et al. 2014), inferring that distribution of cephalosporins in both cerebrospinal fluid and brain intracellular fluid was determined by common contributions of these transporters.

9.3 Drug Delivery to the Central Nervous System

BBB is considered to be a formidable obstacle to drug delivery to the CNS, resulting in ineffectual treatment of neurological disorders and brain cancer. Various attempts have been made to improve CNS drug delivery, including intracerebral implants/intraventricular infusion, opening BBB, inhibiting efflux transporters, and targeting endogenous influx transporters expressed at BBB.

9.3.1 Intracerebral Implants and/or Intraventricular Infusion

Animal experiments have showed that drug directly enters CNS via intracerebral implants or intraventricular infusion to treat brain tumors and epilepsy, but there is also an increase in CNS toxicity associated with increased brain drug concentrations. In human, intracerebral administration was reported to successfully increase delivery of several drugs to the brain, with the possibility of minor systemic toxicity and better effectiveness (Buonerba et al. 2011) for the treatment of malignant glioma. Moreover, these techniques are still invasive, leading to a host of unwanted adverse effects. Their applicability needs further large scale of clinical confirmations.

9.3.2 Transient BBB Disruption

Transient opening of the BBB through intracarotid infusion of hypertonic solutions containing arabinose or mannitol may increase entry of drugs into CNS (Fortin et al. 2004, 2005; Hall et al. 2006). Clinical trials have demonstrated that the osmotic BBB disruption may enhance drug delivery to brain tumors (Fortin et al. 2004, 2005; Hall et al. 2004; Guillaume et al. 2010; Boockvar et al. 2011). Osmotic opening of the BBB using a 25% mannitol solution followed by intracranial intra-arterial infusion of chemotherapeutics has been demonstrated to clinical efficacy on malignant primary or metastatic brain tumors (Angelov et al. 2009; Burkhardt et al. 2012;

Chakraborty et al. 2016; Fortin et al. 2004). However, there are also several drawbacks to this technique. For example, the choice of anesthetic agents and rate of infusion of hypertonic solutions can dramatically affect the degree of BBB opening. Intracarotid administration may be associated with higher ocular and neural toxicity because of the infusion of highly concentrated drugs (Fortin et al. 2004). Transient opening of the BBB also results in undesirable side effects including increases in intracranial pressure, brain edema, or seizures (Bellavance et al. 2008; Burkhardt et al. 2012; Chakraborty et al. 2016; Sanchez-Covarrubias et al. 2014). Animal experiment showed that the focused ultrasound or electrical stimulation might transiently increase BBB permeability and enhance delivery of therapeutics into the brain (Sanchez-Covarrubias et al. 2014), but on clinical practice was reported.

9.3.3 Inhibition of Efflux Transporters

One strategy to improve brain delivery of anticancer drugs is to directly block efflux transporter (such as P-GP and BCRP) transport function at the BBB by using transporter inhibitors. Although a series of P-GP inhibitors have been developed, only a few compounds have been tested for their potential to enhance drug delivery to the brain. The first report that P-GP inhibition could be used to treat brain cancer was a study in nude mice with intracerebrally implanted human U-118 MG glioblastoma (Fellner et al. 2002). In that study, coadministration of valspodar (PSC833) significantly increased paclitaxel levels in brains of mice. Consistent with this issue, paclitaxel alone had no effect on tumor size, but coadministration of valspodar increased paclitaxel brain levels and reduced tumor size by 90%. Similarly, in nude mice with intracerebral implantation of K1735 melanoma or U-87 MG glioblastoma, coadministration of P-GP inhibitor HM30181A (32 mg/kg) with oral paclitaxel (16 mg/kg) biweekly for 28 days also decreased tumor volume of tumors (Joo et al. 2008). Several studies have demonstrated that the P-GP inhibitors cyclosporine A, elacridar (GF120918), tariquidar (XR9576), and zosuquidar (LY335979) increased paclitaxel or docetaxel brain levels (Hubensack et al. 2008; Kemper et al. 2003, 2004a, b).

Importantly, P-GP and BCRP are co-localized at BBB. Moreover, the two transporters have broadly overlapping substrate and inhibitor specificities. Some compounds such as elacridar, cyclosporine A, tariquidar, and valspodar are highly efficient dual BCRP and P-GP inhibitors. Tyrosine kinase inhibitors including erlotinib, gefitinib, imatinib, lapatinib, mesylate, nilotinib, and sunitinib may inhibit BCRP- or PG-mediated drug resistance, some of which are also BCRP and P-GP substrates (Robey et al. 2009). P-GP/BCRP deficiency or usage of dual P-GP and BCRP inhibitors has demonstrated cooperative role of P-GP and BCRP at BBB, inferring that the absence of either P-GP or BCRP alone does not result in an appreciable increase in brain penetration of dual substrates. For example, topotecan is dual P-GP and BCRP substrate. Oral administration of gefitinib increased levels of topotecan in brain extracellular fluid of normal mice penetration (Zhuang et al. 2006)

and in intracellular tumor of mice-bearing orthotopic human U87 or MT330 (Carcaboso et al. 2010). Some tyrosine kinase inhibitors affect the two efflux transporters, especially BCRP via different mechanisms including competitive inhibiting BCRP function and suppressing BCRP expression via inhibiting PTEN/PI3K/Akt signaling pathway (Nakanishi et al. 2006). Thus, a combination of tyrosine kinase inhibitors with other anticancer drugs can have a bimodal effect on ABC transporters, leading to substantial increases in drug levels in brain tumors.

It is notable that direct P-GP or BCRP inhibition improves brain drug delivery of some anticancer drugs and treatment of brain tumors in animal models. Unfortunately, no reports are available on the use of the above mentioned transporter inhibitors in brain cancer patients. Thus, it remains to be demonstrated if the strategy of transporter inhibition can be translated from animal model to patient. In addition, the inhibitions may result in adverse drug reactions due to increased drug concentrations in the brain and other peripheral tissues.

9.4 BBB and CNS-Related Diseases

9.4.1 Brain Inflammation

Brain inflammatory reactions often occur, accompanied by various CNS diseases such as inflammation, ischemia/hypoxia, AD, and infectious disease. In the brain, cell types involved in inflammatory reactions include pericytes, astrocytes, microglia, mast cells, and neurons. Astrocytes exert a critical influence on the BBB phenotype due to their close apposition to the cerebral microvasculature. They help to maintain BBB integrity and immune quiescence through contact-dependent mechanisms or releasing essential soluble factors such as basic fibroblast growth factor (BFGF), TGF-β, GDNF, angiotensinogen, angiopoietin I, and src-suppressed C-kinase substrate. On the other hand, astrocytes activate endothelial cells and impair BBB function via releasing inflammatory cytokines. Neurons and microglia can modulate the barrier phenotype via inducing the expression of immune-related molecules such as chemokines and cell adhesion molecules (CAMs) by BBB endothelial cells (Larochelle et al. 2011).

Some inflammatory modulators may directly increase endothelial permeability and vessel diameter or alter transporter function, together contributing to microvascular leakage and cerebral edema. Some proinflammatory and anti-inflammatory cytokines as well as related molecules such as TNF-α, interferon-γ (INF-γ), TNF-related apoptosis-inducing ligand (TRAIL), tumor necrosis factor-like weak inducer of apoptosis (TWEAK), and interleukins (ILs) (such as IL-1, IL-6, IL-23, IL-17, IL-21, and IL-22) alter BBB permeability via different mechanisms. For example, endothelial cells and astrocytes are considered to be targets of TWEAK where TWEAK acts on the responsive cells and increases BBB permeability and diapedesis (Stephan et al. 2013; Polavarapu et al. 2005) via secreting proinflammatory and chemoattractant cytokines, inducing expression of CAMs

and matrix metalloproteinase 9(MMP-9), activating the MAPK pathway and the NF-κB pathway, or disrupting the tight junction structure.

The activated leukocytes by inflammation may migrate across BBB into the CNS. These processes are involved in interaction of brain microvascular endothelial cells and leukocytes. Moreover, leukocyte migration process itself can alter the characteristics of leukocytes and brain microvascular endothelial cells, favoring neuroinflammation and further facilitating leukocyte migration (Larochelle et al. 2011; Lécuyer et al. 2016).

The peripheral innate immune response to injury leads to the rapid production and local release of proinflammatory cytokines. It was reported that inflammatory pain induced by lambda carrageenan increased BBB permeability via downregulating expression of ZO-1, occludin, and claudin-5 (Campos et al. 2008). The disruption of disulfide-bonded occludin oligomeric assemblies was considered to be also a reason that lambda carrageenan inflammation increased BBB permeability (McCaffrey et al. 2008). The alterations in tight junction protein expression by inflammation seem to be isoform-specific and time-dependent. For example, inflammation induced by complete Freund's adjuvant decreased occludin by about 60% but increased claudin-3 and claudin-5 by 450% and 615%, respectively, without altering expression of ZO-1 and actin (Brooks et al. 2005) at 72 h postinjection. Another study demonstrated that BBB permeability was significantly increased at 24 and 72 h postinjection. Expression of claudin-5 was decreased at 24, while increased at 48 and 72 h postinjection. Significant decrease of occludin expression occurred at 72 h postinjection. Expression of junction adhesion molecule-1 (JAM-1) increased at 48 h and decreased at 72 h postinjection, but ZO-1 was not altered (Brooks et al. 2006).

The HIV virus may break down BBB and enhance infected monocytes across BBB into the brain, producing its CNS effects. HIV-1 gp120 protein was reported to activate signal transducer and activator of transcription 1(STAT1) and induce IL-6 and IL-8 secretion in human brain endothelial cells. IL-6, IL-8, and gp120 also increased monocyte adhesion and migration across in vitro BBB models. These alterations were prevented by the STAT1 inhibitor, fludarabine. These findings indicate that gp120 induces inflammation and BBB dysfunction associated with viral infection through activating STAT1 pathway (Yang et al. 2009).

Bradykinin increases BBB permeability via increasing intracellular [Ca^{2+}], activating phospholipase A2, releasing arachidonic acid, and producing free radicals (Abbott 2000) or accelerating formation of K(ATP) channels (Zhang et al. 2007). In addition, downregulation of ZO-1, occludin, and claudin-5 as well as the rearrangement of F-actin is also involved in increased BBB permeability by bradykinin (Liu et al. 2008a). Elevated levels of plasma homocysteine are often associated with vascular dementia, seizure, stroke, and AD. Homocysteine may increase microvascular permeability via attenuating GABA-A/B receptors and increasing redox stress (Tyagi et al. 2005).

Inflammatory modulators may regulate expression and function of drug transporters at BBB. LPS-induced CNS inflammation downregulates P-GP expression and function in the brain (Goralski et al. 2003) via releasing TNF-α and endothelin-1

or affecting endothelin receptor signaling (Hartz et al. 2006). Dysfunction of BBB by LPS is also dependent on microglia. It was found that in rat brain endothelial cells co-cultured with microglia, LPS lowered P-GP function and induced BBB dysfunction, but these alterations did not occur in rat brain endothelial cells without microglia (Sumi et al. 2010; Matsumoto et al. 2012).

9.4.2 Epilepsy and BBB

9.4.2.1 BBB Permeability Under Epilepsia Status

About 30% of patients with epilepsy do not respond to clinically established antiepileptic drugs, although their plasma concentrations are within the "therapeutic range." One of the plausible hypotheses is alteration in BBB permeability to antiepileptic drugs in patients with medically intractable epilepsy (Aronica et al. 2004; Löscher and Potschka 2002).

Alterations in BBB permeability are dependent on status of epilepsy. Acute seizure or single short seizures disrupt BBB via different mechanisms such as activating MMP-9 (Li et al. 2013b), increases in concentrations of extracellular glutamate (Vazana et al. 2016), and brain inflammation in glia (Librizzi et al. 2012; Lenz et al. 2014), leading to extravasation of IgGs and other serum proteins in the brain (Marchi et al. 2007a; Oztaş et al. 2003; Sahin et al. 2003; Yorulmaz et al. 2013). The accumulation of albumin in the brain has been demonstrated in human epilepsy (Li et al. 2013b; Van Vliet et al. 2007) and epileptic animals (Frigerio et al. 2012; Noé et al. 2016; Van Vliet et al. 2007; Yorulmaz et al. 2013). The extravasation of serum albumin into the brain interstitial space may buffer the antiepileptic drugs due to albumin binding, partly contributing to pharmacoresistance (Salar et al. 2014). Importantly, the brain extravasation of albumin itself induces seizures (Frigerio et al. 2012; Noé et al. 2016; Weissberg et al. 2015; Van Vliet et al. 2007) or increases seizure susceptibility to kainic acid (Frigerio et al. 2012). Moreover, clinical trials and animal experiments have demonstrated that acute BBB opening using intracarotid mannitol causes transient seizures (Burkhardt et al. 2012; Chakraborty et al. 2016; Marchi et al. 2007b; van Vliet et al. 2007), which may be partly attributed to extravasation of albumin. These findings may also partly explain post-injury epilepsy in human (Raabe et al. 2012; Schmitz et al. 2013).

The increases in BBB permeability are often associated with vascular remodeling due to upregulation of vascular endothelial growth factor (VEGF) and its receptors (Rigau et al. 2007). VEGF is known to play a main role in vascular permeability by activating MMPs. The activations of MMPs especially MMP-9 increase BBB permeability via breaking vessel walls and degrading tight junctions (Hayashi et al. 2006; Li et al. 2013b; Zhang and Chopp, 2002). The angiogenic factors upregulated by seizures and the proteolytic processes commonly contribute to the chronic impairment of BBB (Rigau et al. 2007).

9.4.2.2 Overexpression of ABC Transporters under Epilepsia Status

Accumulating evidences have demonstrated overexpression of drug efflux transporters especially P-GP in epileptic tissue (Brandt et al. 2006; Dombrowski et al. 2001; Jing et al. 2010; Liu et al. 2007a; Löscher and Potschka 2002), which contributes to pharmacoresistance to antiepileptic drugs. Some antiepileptic drugs such as phenobarbital (Luna-Tortós et al. 2008; Yang and Liu 2008), lamotrigine (Yang et al. 2008), phenytoin, levetiracetam (Luna-Tortós et al. 2008), and carbamazepine (Sun et al. 2006) were reported to be P-GP substrates. We previously reported that epilepsy induced by pentylenetetrazole significantly increased P-GP expression and function at BBB, accompanied by significant decreases in brain phenobarbital distribution. Treatment with P-GP inhibitor cyclosporine A significantly increased both brain levels and antiepileptic activity of phenobarbital (Liu et al. 2007a). Similarly, coadministration of P-GP inhibitor tariquidar restored the antiepileptic activity of phenobarbital in phenobarbital-resistant rats (Brandt et al. 2006). Epileptogenesis is often accompanied by repetitively temporal hypoxia/ischemia. An in vitro study demonstrated that P-GP overexpression by epilepsy might be partly attributed to repetitive/temporal hypoxia (Liu et al. 2008b). Induction by antiepileptic drugs may also play important roles in overexpression of P-GP at BBB of refractory epilepsy (Wen et al. 2008; Yang et al. 2008). In clinic, pharmacoresistant epilepsy is often gradually developed along with drug treatment. In pentylenetetrazole-kindled epileptic rats, it was found that the kindling significantly increased P-GP activity and expression. Short-term treatment with phenobarbital showed good antiepileptic effect; the maximum effect occurred on day 14 when overexpression of P-GP was reversed. But continuous treatment with phenobarbital had a gradually reduced antiepileptic effect, and on day 40, phenobarbital exhibited no antiepileptic effect, which was accompanied by both a re-enhancement of P-GP expression and decreased phenobarbital concentration in the hippocampus. In age-matched normal rats, chronic treatment with phenobarbital also significantly increased P-GP function and expression in the brain (Jing et al. 2010). These findings indicate that the overexpression of P-GP in the brain is induced by the epileptic seizures during the early stages and by the combined effects of chronic drug treatment and epilepsy at the later stages (Fig. 9.4).

Brain MRP2 is involved in the transport of numerous antiepileptic drugs. Epilepsy was reported to upregulate expression and function of Mrp2 at the BBB of rats, leading to significant decreases in brain distribution of phenytoin. Coadministration of probenecid reversed the decreases in the brain distribution of phenytoin by epilepsy (Yao et al. 2012), indicating that the impairment of drug penetration into the brain may contribute drug resistance in epilepsy.

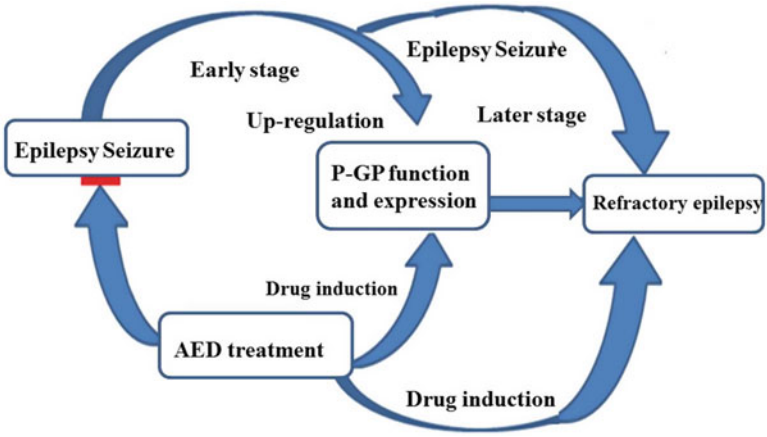

Fig. 9.4 Possible involvement of epilepsy, treatment of antiepileptic drugs (AEDs), and P-GP overexpression in development of refractory epilepsy

9.4.3 Alzheimer's Disease and BBB

9.4.3.1 General Characteristics

Alzheimer's disease (AD) is characterized by cerebrovascular and neuronal dysfunctions leading to a progressive decline in cognitive functions, accounting for the largest proportion (65–70%) of dementia cases in the older population. Pathological hallmarks of AD include neurofibrillary tangles consisting of hyper-phosphorylated microtubule-associated protein called tau and extracellular amyloid plaques. The main component of amyloid plaques in AD brains is amyloid beta (Abeta). Abeta accumulation results in neuronal degeneration via disruption of ionic homeostasis, oxidative damage, inflammatory processes, and disrupting BBB functions (Di Marco et al. 2015; Erickson and Banks 2013). Accumulating evidences have demonstrated a critical role of BBB disruption in the brain Abeta homeostasis via regulating transport of Abeta across BBB. BBB leakage and reduction levels of tight junction proteins further contribute to the pathogenesis of AD (Bell and Zlokovic 2009; Di Marco et al. 2015; Erickson and Banks 2013; Liu et al. 2014).

A long-standing hypothesis for AD is amyloid cascade, which suggests that AD pathology is initiated by the deposition of insoluble Abeta fragments resulting from amyloid precursor protein (APP) proteolysis. The oligomeric Abeta species are the most synaptotoxic forms in AD brains (Shankar et al. 2008). Hippocampal Abeta levels in AD mice were reported to strongly correlate with spatial memory deficits (Zhang et al. 2011). Genetic mutations in the APP may induce AD, but the early-onset AD is rare, less than 1% of all AD cases (Bell and Zlokovic 2009). Moreover, the majority of late-onset AD patients (>99%) do not have mutations increasing APP

processing (Tanzi and Bertram 2005). Other factors such as chronic cerebral hypoperfusion, vascular conditions, atherosclerosis, and diabetes also initiate the neurodegenerative process. For instance, chronic hypoperfusion caused decreases in oxygen, glucose, and other nutrient supply to the brain, directly damaging the parenchymal cells and endothelial cells (Di Marco et al. 2015). BBB dysfunction also mediates a vicious circle in which cerebral perfusion is reduced further and the neurodegenerative process is accelerated (Di Marco et al. 2015).

AD is characterized by early reductions in glucose transport associated with diminished glucose transporter 1(GLUT1) expression at the BBB (Erickson and Banks 2013), leading to impairment of glucose use, subsequently declining in cognitive function. Clinical trial demonstrated correlation of decreases in glucose use in the brain with those cognitive declines (Landau et al. 2011). The brain of AD mice also showed both lower uptake of glucose and expression of Glut1 protein compared with their age-matched control mice (Do et al. 2014; Park et al. 2014a). Further study demonstrated that the reduction of Gut1 expression at BBB worsened AD cerebrovascular degeneration, neuropathology, and cognitive function in mice (Winkler et al. 2015). Moreover, the decreases in Glut1 expression in the brain capillaries of AD mice seemed to be associated with Abeta accumulation (Hooijmans et al. 2007; Sadowski et al. 2004). However, imaging studies of humans demonstrated that glucose use by the brain correlated with ApoE genotype but not with fibrillated Abeta load in the brain (Lehmann et al. 2014; Jagust et al. 2012).

9.4.3.2 AD and Neurovascular Unit

Elementary unit of BBB is a neurovascular unit consisting of neurons, astrocytes, brain endothelium, pericytes, vascular smooth muscle cells, microglia, and perivascular macrophages (Redzic 2011). The functional integrity of the neurovascular unit is essential for normal neuronal and synaptic functioning, which is involved in proper signaling between endothelial cells and pericytes mediated by brain endothelial platelet-derived growth factor B (PDGF-B) and platelet-derived growth factor receptor β (PDGFRβ) in pericytes (Armulik et al. 2010; Bell et al. 2010; Quaegebeur et al. 2010). Roles of pericytes in both BBB development and BBB maintenance have been demonstrated (Winkler et al. 2011; Bell et al. 2010; Hill et al. 2014; Armulik et al. 2010; Quaegebeur et al. 2010). For example, pericyte deficiency induced by deficiency of PDGF-B or PDGFRβ signaling increased BBB permeability in mice (Armulik et al. 2010; Bell et al. 2010; Quaegebeur et al. 2010).

Abeta accumulation and amyloid deposition in cerebral blood vessels may lead to cerebral amyloid angiopathy, which is present in 80% of AD patients (Jellinger 2010; Viswanathan and Greenberg 2011) and strongly associated with cognitive impairment (Attems et al. 2007). AD individuals were reported to possess higher prevalence of cerebral amyloid angiopathy (>80%) compared with the elderly population without AD (10–40%) (Attems et al. 2005; Greenberg et al. 2004). The cerebral amyloid angiopathy is considered to be a major pathological insult to the

neurovascular unit in AD (Thal et al. 2008). Cerebral amyloid angiopathy also caused a rupture of the vessel wall and intracerebral bleeding in about 30% of patients (Cordonnier 2011; Ghiso and Frangione 2002).

The cerebral amyloid angiopathy has been replicated in AD mouse models (Kimbrough et al. 2015; Giannoni et al. 2016; Park et al. 2014a; Yang et al. 2011). In 5xFAD mice, both cerebral amyloid angiopathy and microvascular inflammatory damage occurred concomitantly to PDGFRβ-positive pericytes loss and parenchymal plaque deposition (Giannoni et al. 2016). In hAPP J20 mice, it was found that vascular amyloid displaced the end foot component of the neurovascular unit, in turn, rendering blood vessels rigid and reducing the dynamic range of affected vessel segments (Kimbrough et al. 2015).

Abeta deposition may damage the BBB integrity and potentially lead to spontaneous cerebral hemorrhages via activating MMPs (such as MMP2 and MMP9), later degrading basement membranes, extracellular matrix proteins, and tight junction proteins (Fukuda et al. 2004; Yang et al. 2007; Rosenberg 2009). High levels of reactive oxygen species (ROS) in AD brain may be a mechanism disrupting BBB. Rat experiments also demonstrated that in vivo microdialysis administration of Abeta40 to the brain or intracerebroventricular infusion of Abeta42 directly increased ROS (Kim et al. 2003; Parks et al. 2001), accompanied by decreased in important endogenous antioxidant enzymes related to glutathione-dependent antioxidant response (Kim et al. 2003). Moreover, Abeta can directly lead to neuroinflammation via activating nuclear factor-kappa B (NF-κB)-mediated secretion of proinflammatory cytokines such as TNF-α and IL- 6 (Deane et al. 2003). Clinical investigations have demonstrated that dysfunction BBB in AD patients increased cerebrospinal (CSF)/plasma albumin ratio and that the albumin ratio (CSF/plasma) positively correlated with the severity of medial temporal lobe atrophy (Algotsson and Winblad 2007; Bowman et al. 2007; Matsumoto et al. 2007), although conflicting results have been reported (Erickson and Banks 2013).

9.4.3.3 Abeta Transport in BBB

Transport of Abeta efflux across BBB is involved in various mechanisms including receptor-mediated transcytosis by low-density lipoprotein receptor-related protein-1 (LRP1) and efflux transport mediated by ABC transporters (Fig. 9.5).

LRP1

Within the neurovascular unit, LRP1 is expressed at abluminal side of brain microvessels endothelial cells, vascular smooth muscle cells, pericytes, astrocytes, and neurons. LRP1 internalizes its ligands including apoE-Abeta and directs them to lysosomes for proteolytic degradation. LRP1 also transports its ligands including Abeta, receptor-associated protein, and lipid-free and lapidated apoE from the brain to the blood (Sagare et al. 2012). A series of studies have demonstrated roles of LRP1 in Abeta disposition in the brain. For example, in chronic hydrocephalus rats, the reduced LRP1 levels in brain microvessels are correlated with endogenous Abeta

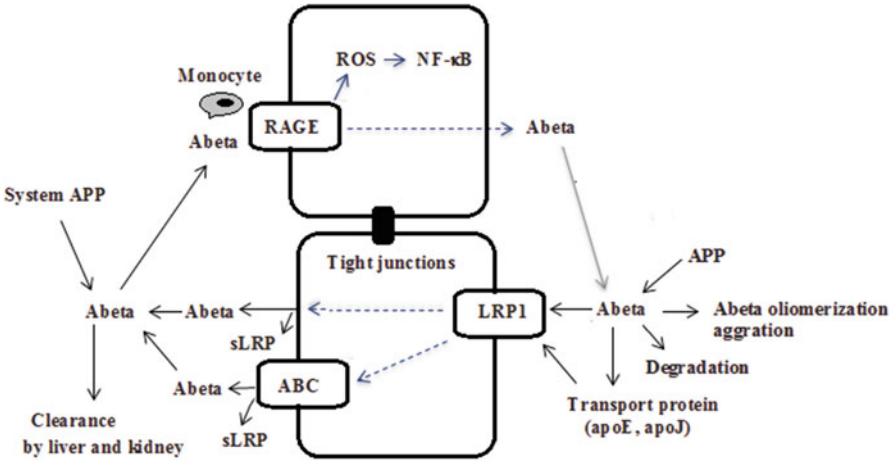

Fig. 9.5 The role of BBB transport in homeostasis of brain Abeta. Influx pathway: RAGE mediates influx of circulating Abeta across the BBB into the brain. RAGE-Abeta interaction leads to generation of reactive oxygen species (ROS) and activation of NF-kB-mediated inflammatory response. RAGE also mediates transport of Abeta-laden monocytes across the BBB. Two efflux pathways. Efflux 1: LRP1 mediates Abeta clearance from the brain via transport of free Abeta and Abeta-apoE2 or Abeta-apoE3 across the BBB into blood. Binding of sLRP to Abeta in plasma prevents RAGE-dependent influx of Abeta. Efflux 2: efflux transport mediated by ABC transporters such as P-GP

deposition (Klinge et al. 2006). The increased Abeta accumulation in the choroid plexus of rats by lead was partly attributed to decreases in expression of LRP1 (Behl et al. 2009, 2010). The downregulation of LRP1 expression by antisenses also reduced BBB clearance of Abeta and increased brain Abeta accumulation, accompanied by impairment of learning ability and recognition memory in mice (Jaeger et al. 2009). The inverse relation between LRP1 levels and Abeta cerebrovascular or brain accumulation has been also demonstrated in AD patients (Shibata et al. 2000; Donahue et al. 2006; Bell et al. 2009). In human, LRP1 expression in brain endothelium often decreases with normal aging (Bell et al. 2009; Deane et al. 2004; Donahue et al. 2006; Silverberg et al. 2010). In addition, Abeta itself decreases LRP1 activity (Owen et al. 2010; Di Marco et al. 2015) via oxidizing LRP-1, in turn, impairing Abeta efflux from the brain and progressively inhibiting its vascular clearance pathway. In plasma, soluble LRP (sLRP) has been identified as a major endogenous peripheral "sink" agent of Abeta. Abeta can bind directly to sLRP, preventing RAGE-dependent influx but enhancing systemic clearance (Sagare et al. 2007). Clinical trial has showed that individuals with AD have slightly lower circulating levels of sLRP-1 and higher levels of oxidized sLRP-1, which markedly lowered its binding affinity to Abeta (Sagare et al. 2007).

ABC Transporters

ABC transporters including P-GP, BCRP, and MRP1 play a pivotal role in controlling Abeta levels in the brain, whose alterations in expression and function

contribute to the aggregation of Abeta in the brain and/or brain endothelial cells, leading to increased risk for developing AD. Lam et al. (2001) first reported Abeta interaction with P-GP; subsequently growing clinical trials have demonstrated roles of P-GP in Abeta clearance from the brain. For example, significant inverse correlation between P-GP expression and the deposition of Abeta in the medial temporal lobe was demonstrated in non-demented subjects (Abuznait and Kaddoumi 2012; Vogelgesang et al. 2002). Similarly, P-GP positive capillaries were reported to inversely correlate with the presence of neurofibrillary tangles and senile plaques in brain tissue of controls and AD patients (Jeynes and Provias 2011; Vogelgesang et al. 2004).

P-GP also impacts formation of cerebral amyloid angiopathy (Vogelgesang et al. 2004), especially capillary cerebral amyloid angiopathy (Carrano et al. 2014). Microvascular expression of P-GP was reported to be strikingly decreased in capillary cerebral amyloid angiopathy vessels but not in AD control samples and in AD cases without capillary cerebral amyloid angiopathy (Carrano et al. 2014). In vitro studies have showed that Abeta decreases P-GP expression (Carrano et al. 2014; Kania et al. 2011) via activating RAGE-NF-κB signaling pathway (Park et al. 2014b). Caffeine intake was found to reduce cognitive decline in aging men and in AD patients (Maia and de Mendonca 2002; Ritchie et al. 2007; van Gelder et al. 2007). Treatment with rifampin also attenuated the rate of cognitive decline in mild to moderate AD patients indexed as SADAScog score (Molloy et al. 2013; Loeb et al. 2004). These findings in human have been confirmed in animal experiments. For instance, deficiency of P-GP has been demonstrated to impair Abeta clearance from the brain of mice (Cirrito et al. 2005; Wang et al. 2016). Tg2576 mice also showed significantly lower expression and function of brain P-GP compared with wild-type mice (Hartz et al. 2010). In these AD mice, treatment with ABCB1 inhibitors or *Abcb1a* knockout further enhanced cerebral Abeta accumulation (Cirrito et al. 2005; Wang et al. 2016). Contrarily, treatment with pregnenolone-16-alpha-carbonitrile, 1α,25-dihydroxyvitamin D3, St. John's Wort extract, or oleocanthal reduced Abeta accumulation in the brain of these AD mice and improved cognition, which may be attributed to restoring expression of P-GP (Brenn et al. 2014; Durk et al. 2014; Hartz et al. 2010; Qosa et al. 2015b). Similarly, caffeine, rifampin, and oleocanthal were also reported to decrease Abeta accumulation in the brain of normal mice via inducing P-GP expression (Arendash et al. 2009; Qosa et al. 2012; Abuznait et al. 2013).

Roles of ABCA1 in Abeta extrusion from the brain were demonstrated by Fukumoto et al. (2002). Their results demonstrated that retinoic acid, 22(R)-hydroxycholesterol, and TO-901317 significantly increased secretion of Abeta from neuronal cells via induction of Abca1. Blockading Abca1 expression with Abca1 RNAi reversed the increased secretion of Abeta induced by retinoic acid or 22(R)-hydroxycholesterol (Fukumoto et al. 2002). Unlike ABCB1, ABCA1 does not directly transport Abeta. For instance, Abca1 deficiency did not alter the elimination

of cerebral microinjected ^{125}I-Abeta40 across BBB of mice (Akanuma et al. 2008). In general, ABCA1 regulates both the level of apolipoprotein E (ApoE) and its state of lipidation via regulating neuronal cholesterol efflux to apoE. apoE also acts as a chaperone for Abeta by binding the peptide and altering its conformation, indicating that ABCA1 regulates Abeta transport in Abeta-apoE. In line with this, *Abca1*$^{-/-}$ mice exhibited substantial reductions in levels of apoE-containing lipoproteins and total level of apoE protein in the brain (Wahrle et al. 2004), which is likely due to reduced apoE secretion from both astrocytes and microglia or a diminished pool of intracellular apoE in microglia (Hirsch-Reinshagen et al. 2004). In AD mice, *Abca1* deficiency increased amyloid deposition in parallel with reduced apoE levels (Koldamova et al. 2005; Lefterov et al. 2009; Wahrle et al. 2005) and also exacerbated cognitive performance (Lefterov et al. 2009). On the contrast, overexpression of Abca1 decreased brain Abeta deposition and increased lipidation of apoE-containing particles (Wahrle et al. 2008).

The role of BCRP in Abeta extrusion at the BBB was demonstrated in *Abcg2*$^{-/-}$ mice. The results showed that compared with wild-type mice, *Abcg2*$^{-/-}$ mice showed higher fluorescence intensity in the brain following intravenous injection of Cy5.5-labeled Abeta1–40 peptides, suggesting that BCRP acts as a gatekeeper at the BBB to prevent blood Abeta from entering into the brain (Xiong et al. 2009). The in vivo finding was further confirmed by in vitro studies utilizing cells overexpressing BCRP (Xiong et al. 2009; Do et al. 2012). In situ brain perfusion also demonstrated that GF120918 strongly enhanced the uptake of [^{3}H] Abeta1-40 by the brains of *Abcb1*$^{-/-}$ mice, but not by the brains of *Abcb1*$^{-/-}$:*Abcg2*$^{-/-}$ mice (Do et al. 2012). Unlike *ABCB1*, *ABCG2* gene and protein were significantly upregulated in the brains of AD and cerebral amyloid angiopathy patients compared to control subjects (Xiong et al. 2009). The upregulation of Bcrp was also observed in AD mice (Do et al. 2016; Xiong et al. 2009). The BCRP upregulation is considered as a compensatory mechanism initiated by the pathological microenvironment in the neurovascular unit (Xiong et al. 2009) or as a protective mechanism (Shen et al. 2010). However, some reports showed that BCRP expression in the brain vasculature of AD patients was comparable to normal cases (Wijesuriya et al. 2010) or decreased in capillary cerebral amyloid angiopathy cases (Carrano et al. 2014). Moreover Abeta did not alter BCRP expression in vitro BBB model (Carrano et al. 2014; Kania et al. 2011). These results clearly indicate the need for further investigations about the role of BCRP in the pathology of AD.

4-hydroxy-2-transnonenal is linked to AD (Sultana and Butterfield 2004; Butterfield et al. 2002). Significant increase in free 4-hydroxy-2-transnonenal has been demonstrated in the cerebrospinal fluid, amygdala, hippocampus, and parahippocampal gyrus in the brain of AD patients (Lovell et al. 1997; McGrath et al. 2001). 4-Hydroxy-2-transnonenal is an endogenous substrate of MRP1, indicating that MRP1 is involved in AD process. MRP1 is also involved in in cerebral Abeta clearance and brain accumulation (Krohn et al. 2011). In AD mice, *Abcc1* deficiency substantially increased cerebral Abeta levels. In contrast, Mrp1 inducers

thiethylperazine and St. John's wort extracts significantly reduced Abeta levels in the brain of AD mice (Hofrichter et al. 2013; Krohn et al. 2011). Moreover, administration of John's wort extracts improved cognitive performance (Hofrichter et al. 2013).

Other ABC transporters such as ABCA2, ABCA7, ABCG1, and ABCG4 may be involved in pathology of AD (Abuznait and Kaddoumi 2012; Pahnke et al. 2014). For example, a meta-analysis in African-American participants showed that variants in ABCA7 gene were strongly associated with the increased risk for late-onset AD (Reitz et al. 2013). *Abca7* deficiency altered brain lipid profile and impaired memory compared with wild-type mice. In AD mice, *Abca7* deficiency exacerbated brain Abeta deposition via facilitating the processing of APP and Abeta production through increasing the levels of β-secretase 1 without affecting Abeta clearance (Sakae et al. 2016).

RAGE

Receptor for advanced glycation end products (RAGE) is a multiligand receptor of the immunoglobulin superfamily. Within CNS, RAGE is expressed on the cell surface of vascular endothelial cells, pericytes, smooth muscle cells, neurons, and glial cells, acting as a receptor for Abeta (Sagare et al. 2013). At the BBB, RAGE mediates transport of circulating Abeta into the brain, where RAGE-Abeta interaction also results in NF-κB-dependent endothelial cell activation, neuroinflammatory response, and monocyte trafficking across the BBB (Sagare et al. 2013; Deane et al. 2003, 2009). RAGE also mediates Abeta-induced neurotoxicity and inflammatory response via causing oxidant stress and activating microglia. Increases in RAGE expression have been demonstrated in AD brains (Choi et al. 2014; Lue et al. 2001; Sasaki et al. 2001; Miller et al. 2008). The overexpression of RAGE exacerbated impairment of memory and of synaptic plasticity and alteration in neuropathologic markers in AD mice, while suppression of RAGE expression prevented memory impairment and diminished neuropathologic changes in AD mice (Arancio et al. 2004; Cho et al. 2009). These results indicate that RAGE is a facilitator for Abeta-induced neuropathologic changes and that RAGE is a potential therapeutic target in AD.

FPS-ZM1, a RAGE-specific inhibitor, was reported to effectively control progression of an Abeta-mediated brain disorder and the related neurovascular and cognitive dysfunction in AD mice (Deane et al. 2012). Similarly, intraperitoneal administration of FPS-ZM1 significantly reduced the upregulation of Abeta production, inflammation, and oxidative stress and increased escape latency of rats in the Morris water maze test by intrahippocampal injection of advanced glycation end products (Hong et al. 2016). Phase 1 and phase 2 clinical trials demonstrated that 5 mg/day TTP448 (a RAGE-specific inhibitor) had a positive effect in slowing down cognitive decline as compared to placebo (Walker et al. 2015), which needs further investigation.

9.4.4 Parkinson's Disease and BBB

Parkinson's disease (PD) is the second most common neurodegenerative disease and characterized by loss of dopaminergic cells in the substantia nigra pars compacta and presence of Lewy bodies. An integral characteristic of Lewy bodies is that they are mainly comprised of the protein α-synuclein (Duda et al. 2000). Functions of α-synuclein at the neuronal synapse include vesicular stabilization, synaptic pool maintenance, regulation of dopamine synthesis, and a potential role in synaptic plasticity (Bates and Zheng 2014). Importantly, either decreases or increases in α-synuclein expression leads to the neurodegeneration in PD. For example, mutations in the α-synuclein gene can cause familial PD, and multiplication of the normal wild-type α-synuclein gene can also increase the risk of sporadic PD (Kanaan and Manfredsson 2012).

Within the brain, α-synuclein can be released by neurons to the interstitial fluid or CSF via exocytosis mechanism and also absorbed into neurons from extracellular fluids via endocytosis mechanism. The extracellular α-synuclein is involved in the progression of PD. α-synuclein is cleared from CSF by the choroid plexus or by drainage from the subarachnoid spaces to the blood. Astrocytes also uptakes α-synuclein via endocytosis, leading to cellular toxicity including the death of afflicted astrocytes (Bates and Zheng 2014).

Aggregation of α-synuclein and ensuing development of α-synuclein fibrils precede the development of Lewy bodies and PD. The aggregation of α-synuclein in PD brain may be due to (1) the increased production of α-synuclein by neural processes; (2) the interneuronal transmission of toxic α-synuclein aggregated species; (3) decrease in clearance from the brain; (4) increase in influx from the blood into the brain; or (5) a combination of the these concepts (Bates and Zheng 2014; Matsumoto et al. 2017).

α-Synuclein transports across the BBB in bidirectional manner. α-Synuclein could readily transport from the brain to blood in exosomes (Sui et al. 2014; Shi et al. 2014); however, the efflux was not inhibited by unlabeled α-synuclein, Abeta1-42, and P-GP inhibitor cyclosporine A. In general, the plasma levels of α-synuclein exceed those of CSF by about tenfold (Hong et al. 2010). Peripheral α-synuclein enters the brain across BBB (Sui et al. 2014) via adsorptive-mediated transcytosis (Matsumoto et al. 2017), becoming a source of potentially pathogenic α-synuclein. LPS increases the blood-to-brain uptake of α-synuclein via disrupting BBB or enhancing adsorptive-mediated transcytosis (Sui et al. 2014; Matsumoto et al. 2017). LPS is a product of gut microbiota, which upregulates local and systemic inflammation. These findings may partly explain contributions of brain-gut-microbiota axis to PD development (Mulak and Bonaz 2015).

The pathogenesis of PD is often linked to BBB disruption, in turn, further worsening PD progression (Kortekaas et al. 2005; Stolp and Dziegielewska 2009; Weiss et al. 2009). BBB dysfunction has also been demonstrated in PD patients (Faucheux et al. 1999; Kortekaas et al. 2005) and in PD animal models (Carvey et al. 2005; Vautier et al. 2009; Zhao et al. 2007). Rat experiments have also showed apparent correlation between BBB disruption and dopaminergic neuronal loss in

substantial nigra (Rite et al. 2007; Villarán et al. 2009). Similarly, neurotoxicant MPTP resulted in both impairment of BBB function and loss of dopaminergic neurons in substantia nigra via activating MMP-3 (Chung et al. 2013). Moreover, environmental exposures to neurotoxicants are considered to be risk factors for PD (Kamel 2013; Wang et al. 2014; van der Mark et al. 2012), and pesticides themselves disrupt BBB permeability (Gupta et al. 1999). These findings indicate that BBB dysfunction contributes, at least in part, to the pathogenesis of PD and may explain why exposures to neurotoxicants (such as MPTP) result in the development of a progressive PD.

It is widely accepted that P-GP at the BBB protects the brain from toxic substances circulating in the blood, showing important roles of P-GP in the development of neurodegenerative diseases, including PD. P-GP expression or activity at the BBB varies with age, disease progression, and *ABCB1* pharmacogenomics (Miller 2010). Some neurotoxicants such as diazinon, MPP^+, and rotenone are P-GP substrates (Lacher et al. 2015).

The *ABCB1* gene SNPs, alone or in conjunction with exposure to insecticides and pesticides, have been suggested to be important risk factors for PD development in different populations (Dutheil et al. 2010; Lee et al. 2004; Tan et al. 2005; Westerlund et al. 2009; Zschiedrich et al. 2009). For example, genetic variants at *c.3435C/T* and *c.2677G/T/A* in *ABCB1* and occupational exposures to organophosphorus pesticides or organochlorines increased risk of PD (Dutheil et al. 2010; Narayan et al. 2015). Similarly, *ABCB1* variant at *c.3435C/T* also showed a trend to increase the risk to develop PD in conjunction with exposure to pesticides (Zschiedrich et al. 2009). A meta-analysis showed the associations between the *ABCB1* polymorphisms and PD. Significant association was noticed for *c.1236C/T* polymorphism and PD risk. Further, ethnicity-based analysis showed significant association for *c.1236C/T* in allelic model of Asian population and also in the recessive models of both Asian and Caucasian populations. However, insignificant associations were noticed for *c.3435C/T* in all the four models (Ahmed et al. 2016). Haplotype analysis also revealed significant association of the *1236C-2677G* haplotype with PD and a trend toward association with disease of the *1236C-2677G-3435C* haplotype (Westerlund et al. 2009). In ethnic Chinese in Hong Kong, it was found that haplotypes containing *c.2677G/T/A* and *c.3435C/T* were significantly associated with risk of PD. In particular, the *2677T-3435T* haplotype was strongly associated with a reduced risk of PD, indicating that an MDR1 haplotype containing SNPs *2677T* and *3435T* protects against PD in ethnic Chinese (Tan et al. 2005). A novel and heterozygous DNA sequence variant *g.117077G>A* was identified in one PD patient but not in controls, indicating the novel variant may contribute to PD development as a rare risk factor (Li et al. 2014). However, no significant association between *ABCB1* gene SNPs and PD has been found in some studies, though frequencies of the *ABCB1* gene SNPs are higher in PD patients than controls (Droździk et al. 2003; Funke et al. 2009; Furuno et al. 2002; Tan et al. 2004). PET data using (R)-[^{11}C]-verapamil also demonstrated that P-GP function was not altered in BBB of early-stage PD patients (Bartels et al. 2008a) but decreases in P-GP function occurred at later disease stages PD (Bartels et al. 2008b). Therefore, association of P-GP function and PD needs further investigation.

9.4.5 Amyotrophic Lateral Sclerosis and BBB

Amyotrophic lateral sclerosis (ALS) is a severe neurodegenerative disease with a complicated pathogenesis, characterized by progressive motoneuron degeneration in the brain and spinal cord leading to muscle atrophy, paralysis, and death typically within 3–5 years from diagnosis. Most ALS cases are sporadic ALS with only 5–10% genetically linked familial ALS. About 20% of familial ALS cases show missense mutations in the Cu/Zn superoxide dismutase1 (SOD1) gene. The transgenic rodents expressing mutant human SOD1 develop progressive motoneuron degeneration and clinical signs that closely mimic human ALS (Vinsant et al. 2013). Although the exact mechanism responsible for motoneuron degeneration in ALS is not fully understood, one possible pathogenic mechanism is a neurovascular disease which is attributed to impairment of BBB and blood-spinal cord barriers (BSCB). Structural and functional BBB/BSCB impairments have been demonstrated in ALS patients using analysis of albumin and other serum-derived proteins in CSF and measurements of albumin CSF/serum ratios (Brettschneider et al. 2006; Garbuzova-Davis et al. 2011; Garbuzova-Davis and Sanberg 2014) or in autopsied tissue from ALS patients (Henkel et al. 2009; Garbuzova-Davis et al. 2011; Winkler et al. 2013). The increases in perivascular, intraparenchymal lymphocytic infiltration, cytokines, local inflammatory/immune cell activation, extravasation of erythrocytes, and the reduces in capillary blood flow have been also detected in the spinal cord of ALS patients, which are associated with motoneuron death (Boillée et al. 2006; Garbuzova-Davis et al. 2011; Rule et al. 2010; Winkler et al. 2013; Sasaki 2015). Consistent with findings in humans, transgenic rodents expressing mutant human SOD1 also develop a spontaneous BSCB breakdown, characterized by vascular leakage and downregulation of tight junction protein (such as ZO-1, occludin, and claudin-5) expressions (Zhong et al. 2008). The BSCB breakdown was also accompanied by other alterations such as extravasation of erythrocytes, downregulation of Glut1 and CD146 expressions, decreased laminin content of the basement membrane in capillaries, ultrastructural alterations in the vessels surrounding degenerating neurons in the brain and spinal cord, and decreases in regional cerebral blood flow or spinal blood flow (Garbuzova-Davis et al. 2007a, b, 2011; Ishikawa et al. 2007; Nicaise et al. 2009a, b; Zhong et al. 2008). It was reported that SOD1 transgenic mice demonstrated 10–15% reductions in total capillary length and 30–45% decreases in spinal cord blood flow (Zhong et al. 2008) compared with control mice. Importantly, these alterations often occur prior to motoneuron loss and inflammatory changes (Miyazaki et al. 2011, 2012; Zhong et al. 2008). Moreover, the decreases in spinal blood flow are closely related to the decrease in capillary diameter, density, and red blood cell velocity (Miyazaki et al. 2012; Zhong et al. 2008) and also become progressively larger with disease progression. Chronic hypoperfusion and motoneuron injury by the reduced blood flow are partly attributed to increases in expression of hypoxia-inducible factor-1α and VEGF (Murakami et al. 2003; Xu et al. 2011).

BSCB breakdown also causes extravasation of erythrocytes, microhemorrhages or hemosiderin deposits, preceding motor symptoms, and neuronal loss. Cervical cords from ALS patients were reported to possess perivascular deposits of erythrocyte-derived hemoglobin and hemosiderin typically 10–50 μm in diameter suggestive of erythrocyte extravasation. Perivascular hemoglobin deposits in ALS patients were reported to increase to 3.1-folds of controls (Winkler et al. 2013). A report showed the accumulation of hemoglobin and iron in the spinal cord of ALS mice, leading to early motoneuron degeneration via iron-dependent oxidant stress, which was confirmed by the fact that administration of warfarin accelerated microvascular lesions. Moreover, the motoneuron dysfunction and injury were proportional to the degree of BSCB disruption at early disease stages. Early treatment with an activated protein C analog delayed onset of motoneuron impairment and degeneration via restoring BSCB integrity and eliminating hemoglobin and iron deposits. Similarly, early treatment with chelation of blood-derived iron or antioxidants also mitigated early motoneuronal injury. These findings demonstrated roles of hemoglobin and iron accumulation due to BSCB breakdown in early motoneuron degeneration in ALS mice (Winkler et al. 2014)

Several evidences have showed that direct SOD1 damage within motoneurons is a central component of driving disease initiation but not disease progression (Alexianu et al. 2001; Stoica et al. 2016; Yamanaka et al. 2008), whereas progression is predominantly determined by responses within microglia and astrocytes (Haidet-Phillips et al. 2011; Meyer et al. 2014; Qian et al. 2017; Stoica et al. 2016; Yamanaka et al. 2008). Alexianu et al. (2001) investigated immune-inflammatory factors in the destruction of motor neurons in ALS mice at different ages (40, 80, and 120 days). The result showed that the earliest change observed was the upregulation of intercellular cell adhesion molecule-1(ICAM-1) in the ventral lumbar spinal cord of 40 days. IgG and its receptor for Fc portion reactivities were detected on motor neurons as early as 40 days and on microglial cells at later stages. Microglial activation was first evident in the ventral horn at 80 days, whereas reactive astrocytes and T cells became most prominent in 120 days (Alexianu et al. 2001). In vitro study showed that the expression of mutated human SOD1 in primary mouse spinal motoneurons did not provoke motoneuron degeneration. Conversely, rodent astrocytes expressing mutated SOD1 killed spinal primary and embryonic mouse stem cell-derived motoneurons (Nagai et al. 2007). Microglial activation has been demonstrated in tissues from ALS patients (Brettschneider et al. 2012; Corcia et al. 2012). A PET study demonstrated significant increase of distribution of volume ratios of 18F-DPA-714 in tissues from ALS patients, inferring microglial activation (Corcia et al. 2012). Microglial pathology (as depicted by CD68 and Iba1) was significantly more extensive in the corticospinal tract of ALS cases with a rapid progression of disease. The scores on the clinical upper motoneuron scale were significantly related to microglial pathology in the cervical corticospinal tract (Brettschneider et al. 2012). The functions of activated microglia are better represented as continuum between two extreme activation states: classically activated microglia and alternatively activated microglia. The classically activated microglia are neurotoxic microglial phenotypes

due to their secretion of ROS, proinflammatory cytokines, and increased levels of dihydronicotinamide-adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) expression (marker of toxic phenotypes). The alternatively activated microglia are neuroprotective microglial phenotypes, which block proinflammatory response and produce high levels of anti-inflammatory cytokines and nueotrophic factors (Liao et al. 2012). Microglia activation was also readily apparent in ALS mice (Alexianu et al. 2001; Henkel et al. 2004; Liao et al. 2012; Beers et al. 2011). The neuroprotective microglial phenotype was switched to the neurotoxic microglial phenotype, which was associated with the transition of ALS mice from a stable to a rapidly progressive phase of disease (Liao et al. 2012; Beers et al. 2011). The increased expression of the dendritic cell markers was also reported to be associated with a more rapidly progressing disease in ALS patients (Henkel et al. 2004). T lymphocytes are able to cross into the brain and spinal cord parenchyma, where they interact with resident microglia, inducing them to adopt either a cytotoxic or protective phenotype, depending on the stage of disease. In ALS mice, motor weakness begins in the hind limbs of mice and slowly progresses to the forelimbs. A report demonstrated that the protective phenotypes increased in cervical cords and lumbar cords of ASL mice as disease progresses, but they increased at a more rapid rate in the cervical cords than in the lumbar cords. However, cytotoxic phenotypes were similar in both regions at disease onset (11 and 16 weeks) and then increased as the disease progresses, but lumbar spinal cords showed more marked increases compared with lumbar cords (Beers et al. 2011). Moreover, neuroprotective markers including brain-derived neurotrophic factor (BDNF), GDNF, and IL-4 were also increased in the cervical region compared with the lumbar region of ALS mice, but the toxic markers tumor necrosis factor alpha (TNF-α), IL-1β, and NOX2 were not different between ALS mice cervical and lumbar regions (Beers et al. 2011). T lymphocytes were also infiltrated into lumbar spinal cords of ALS mice prior to the cervical region. These findings indicate that augmented protective responses in the cervical spinal cords may partly explain the delayed forelimb motor weakness in ALS mice (Beers et al. 2011). Data from microglia of ALS mice at disease onset (11 weeks) demonstrated that the levels of Ym1 (chitinase 3-like 3), CD163 and BDNF mRNA were similar to those of wild type mice, but levels of NOX 2 mRNA were significantly increased compared with wild type mice. Whereas, at end-stage disease, levels of Ym1, CD163, and BDNF mRNA in microglia from ALS mice were remarkably decreased. Expression of NOX 2 mRNA was still increased, and the extent of increase was greatly larger than that at disease onset. These results were in line with the activated microglial phenotypes. More importantly, when co-cultured with motoneurons, microglia of ALS mice at disease onset were neuroprotective and enhanced motoneuron survival than co-cultured microglia of ALS mice at end-stage disease, indicating that microglia of ALS mice at end-stage disease were toxic to motoneurons (Liao et al. 2012).

Platelet-derived growth factor C (PDGF-C) pathway is also involved in BSCB dysfunction by ALS. The secreted latent PDGF-C protein is cleaved to an active ligand by the tissue plasminogen activator (tPA) (Fredriksson et al. 2004). It was reported that motor neurons of both human sporadic ALS patients and ALS mice

showed higher gene expression of PDGF-C and its activator tPA gene (Lewandowski et al. 2016). PDGF-C expression was increased at the presymptomatic stage and accumulated in parenchyma during onset and symptomatic stages in spinal cords of ALS mice. The vessels displaying BSCB dysfunction were surrounded by perivascular astrocytes expressing the receptor for PDGF-C (PDGFRα), indicating that the presymptomatic activation of the PDGF-C pathway in ALS mice led to BSCB dysfunction. Decreasing PDGF-C expression in ALS mice using knockout of its gene or treatment with PDGFRα inhibitor imatinib restored vascular barrier properties, reduced motoneuron loss, and delayed symptom onset. The correlation between the decreased expression of PDGF-C or PLAT and the delayed onset age in sporadic ALS patients suggests that this mechanism is common between familial and sporadic disease and clinically relevant. But, neither PDGF-C inhibition nor restoration of BSCB integrity prevented capillary regression at disease end stage and extended survival (from birth to critical endpoint). The degree of vessel regression at end stage of ALS mice positively correlated with more aggressive disease after onset regardless of BSCB status (Lewandowski et al. 2016).

Pericytes play important roles in the BSCB and the BBB. A clinical trial showed a 54% reduction in pericyte number in ALS patients compared to controls. The pericyte reduction also correlated positvely with the magnitude of BSCB damage in ALS patients (Winkler et al. 2013). The highest percentages of the capillary wall covered by pericytes were found in the brain regions (about 80%), followed by thoracic (68~66%), cervical (59~55%), and lumbar (52~48%) spinal cord anterior horn of mice. In line with this issue, vascular leakages to 40,000 Da dextran and 150,000 Da dextran positively correlated to reductions in percentage of pericyte coverage (Winkler et al. 2012). The pericyte-deficient mice showed lower percentage of pericyte coverage in cervical, thoracic, and lumbar spinal cord than wild-type mice (Winkler et al. 2012). The pericyte degeneration was also reported to be associated with progressive microvascular degeneration, reduction in expression of tight junction proteins (ZO-1 and occludin) and in the capillary basement membrane proteins collagen IV, vascular laminin, loss of motor neurons or accumulation of the endogenous plasma proteins (such as IgG, fibrinogen and fibrin) (Bell et al. 2010; Winkler et al. 2012). Treatment with intraperitoneal pericytes extended significantly survival in ALS mice via stimulating the host antioxidant system (Coatti et al. 2017). In vitro data showed that co-cultured pericytes strengthened the barrier integrity in primary cultures of rat brain endothelial cells, characterized by the increased electrical resistance and the decreased BBB permeability (Nakagawa et al. 2007; Hayashi et al. 2004). Moreover, co-cultured pericytes also restored hypoxia-induced BBB disruption (Hayashi et al. 2004).

Several studies have demonstrated upregulation of P-GP expression in the spinal cord of ALS mice (Boston-howes et al. 2008; Milane et al. 2010; Jablonski et al. 2012, 2014; Chan et al. 2017) and ALS patients (Jablonski et al. 2012, 2014), accounting for the therapeutic failure of nordihydroguaiaretic acid and riluzole. For example, riluzole brain disposition in the brain of ALS mice was 1.7-fold lower compared to control mice, and coadministration of P-GP inhibitor minocycline significantly increased riluzole brain disposition in ALS mice (Milane

et al. 2010), which might partly explain the findings that riluzole treatment with minocycline showed a higher efficacy in animal models than riluzole alone (Kriz et al. 2003). Similarly, P-GP knockout or coadministration of P-GP inhibitor elacridar also increased riluzole brain disposition in ALS mice and significantly extended survival compared to control/placebo and riluzole/placebo group (Jablonski et al. 2014). These findings indicate that drug combinations with efflux pumps inhibitors may improve treatment efficacy.

9.4.6 Diabetes Mellitus and BBB

Diabetes mellitus is a systematic metabolic disease, which develops a number of well-recognized vascular complications. It is widely accepted that diabetes may induce BBB dysfunction (Dai et al. 2002; Starr et al. 2003; Liu and Liu 2014), disrupting homeostasis and contributing to long-term cognitive and functional deficits of the CNS. However, studies whether diabetic condition affects BBB permeability to drugs often give contrary results. These discrepancies may be due to the type and degree of diabetes, or experimental condition. For example, a clinical report showed that IgG and albumin levels in brains of diabetic patients (type II and type I) and control subjects were similar using corresponding specific antibodies (Dai et al. 2002). But increases in BBB permeability to gadolinium-diethylenetriamine pentaacetic acid were found in type II diabetic patients using gadolinium magnetic resonance imaging (Starr et al. 2003). In diabetic rats, it was reported that cerebral occludin content was significantly reduced, but the ZQ-1 content in cerebral tissue of diabetic rats was not significantly altered (Chehade et al. 2002). Increased MMP activity, especially MMP-9, was also found in diabetic patients (Ju et al. 2015).

Cerebral edema, usually vasogenic edema, is a devastating complication of diabetic ketoacidosis, partly resulting from increase in BBB permeability (Tasker and Acerini 2014; Vavilala et al. 2010). The increases in BBB permeability by diabetic ketoacidosis may be involved in various factors including hyperglycemia, the increased MMP activities, the elevated levels of ketone bodies, and neuroinflammation. For example, in diabetic rats induced by streptozotocin, the increased permeability to sucrose was reported to be associated with decreased production of tight junction proteins (occludin and ZO-1) and the increase of plasma MMP activity (Hawkins et al. 2007a). In accordance, diabetic mice by streptozotocin showed higher MMP-9 activity and expression and lower expression of tissue inhibitor of matrix metalloproteinase-1. The diabetic mice also showed the increased water content and sodium level in both the cortex and hippocampus (Aggarwal et al. 2015). In children with diabetic ketoacidosis, circulating MMP-2 levels decreased and MMP-9 levels increased compared with children without diabetic ketoacidosis (Garro et al. 2017). Similarly, plasma MMP-8 and MMP-9 concentrations correlated with diabetic ketoacidosis severity (Woo et al. 2016a). Neuroinflammation may be

involved in the BBB disruption by diabetic ketoacidosis. In the fatal brain edema associated with diabetic ketoacidosis, morphological and functional changes in the BBB were found manifested as disruption/absence of TJ proteins in the parenchymal brain microvessels and extravasation of albumin. The morphological changes of BBB integrity were accompanied by astrocyte and microglia activation and the increased expression of chemokine ligand 2, NF-κB, and nitrotyrosine, markers of neuroinflammatory injury (Hoffman et al. 2009). In HBMECs, it was found that VEGF induced IL-8 expression and enhanced leukocyte infiltration through the expression of chemokines, such as IL-8 (Lee et al. 2002). Release of destructive polymorphonuclear neutrophil azurophilic enzymes increased BBB permeability. The children with acute diabetic ketoacidosis showed significantly elevated polymorphonuclear neutrophils and plasma azurophilic enzymes (including human leukocyte elastase, proteinase-3, and myeloperoxidase), but only proteinase-3 levels significantly correlated with diabetic ketoacidosis severity. In HBMECs, it was found that proteinase-3 degraded both occludin and VE-cadherin, leading to increase in cell permeability, which demonstrated roles of proteinase-3 in vasogenic edema induced by diabetic ketoacidosis (Woo et al. 2016b).

High glucose and ketone bodies themselves impair BBB via different mechanisms. In vitro studies showed that β-hydroxybutyrate increased expression of VEGF, while acetoacetate increased expression of endothelin-1 (Isales et al. 1999) and ICAM-1 (Hoffman et al. 2002). Glucose concentrations of 10 and 30 mM, but not 50 mM, also resulted in increased expression of ICAM-1 in vitro BBB model (Hoffman et al. 2002). Hyperglycemia also significantly compromised the BBB integrity via activating protein kinase C (PKC)-β signal pathway (Shao and Bayraktutan 2013) which was attenuated by selective inhibitor of PKC-β, specific NADPH oxidase inhibitor, antioxidants (vitamin C and free radical scavengers), and antioxidant enzymes (catalase and superoxide dismutase) (Allen and Bayraktutan 2009; Shao and Bayraktutan 2013). Moreover, hyperglycemia also induced brain microvascular endothelial cell apoptosis via activating PKC-β signal pathway and consequently stimulated oxidative stress related to NADPH oxidase (Shao and Bayraktutan 2014).

Diabetic patients are often accompanied by hypertension. Acute hypertension in diabetic hypertensive rats was reported to increase the BBB permeability (Awad 2006; Kaya et al. 2003); treatment with losartan (Kaya et al. 2003) or candesartan (Awad 2006) ameliorated the increases in BBB permeability induced by hypertension. Diabetes elicit a progressive impairment of neuronal function such as cognitive impairment, stroke, cerebral ischemia, and schizophrenia (Prasad et al. 2014; Serlin et al. 2011). Although the association of BBB breakdown with diabetes-induced cognitive impairment is not conclusive (Mogi and Horiuchi 2011), growing clinical and experimental evidences have demonstrated that neurovascular endotheliopathy and BBB hyperpermeability occur in schizophrenia patients (Najjar et al. 2017) or major depressive disorder (Najjar et al. 2013). The neurovascular endotheliopathy and BBB hyperpermeability are considered to be linked to oxidative stress and neuroinflammation (Najjar et al. 2013, 2017).

Another alteration of BBB function is the alterations in expression and function of some drug transporters, especially ABC transporters, in cerebral microvessels (Liu and Liu 2014). For example, expression and function of P-GP in the BBB of diabetic rats by streptozotocin were downregulated, resulting in increase of vincristine and rhodamine 123. These alterations by diabetes were attenuated by insulin treatment (Liu et al. 2006, 2008d). In in vitro BBB model, insulin upregulated P-GP expression and function via PKC/NF-κB pathway (Liu et al. 2009), further confirming roles of insulin in P-GP expression and function at BBB. Diabetes also downregulated BCRP expression and function at BBB of rats, causing increases in brain distribution of prazosin and cimetidine (Liu et al. 2007c). It was in contrast to findings in P-GP (Liu et al. 2008a, 2009); insulin suppressed the function and expression of BCRP in in vitro BBB model (Liu et al. 2011), inferring that the mechanisms leading to impairment of activity and expression of BCRP by diabetes were different from those of P-GP. Unlike P-GP and BCRP, expression of MRP-2 protein in cerebral microvessels of rats was regulated by diabetes (Hawkins et al. 2007b).

Accumulation of Abeta in the brain is considered to be a cause of AD. It is demonstrated that P-GP, possibly BCRP, is involved in the transport of Abeta across the BBB, indicating that downregulation of P-GP function and expression by diabetes affected Abeta accumulation in the brain. Our previous report demonstrated that Abeta accumulation in the brain of diabetic rats was partly attributed to decrease in efflux from the brain and increase in influx from the blood to the brain (Liu et al. 2008c), which partly explained contribution of diabetes to the pathogenesis of AD. The absence of functional P-GP at the BBB leads to highly increased brain penetration of a number of important drugs, inducing dramatically increased neurotoxicity, or fundamentally altered pharmacological effects of the drugs on CNS. Phenobarbital, an antiepileptic and sedative drug, is a substrate for P-GP. It was reported that the impaired function and expression of P-GP at BBB of diabetic mice increased brain distribution and increased its CNS activity/toxicity. The diabetic mice showed significantly longer duration of phenobarbital-induced loss of the righting reflex and shorter latency time of loss of the righting reflex compared to normal mice. All the diabetic mice died after 5 h following intravenous administration of phenobarbital (100 mg/kg) (Liu et al. 2007b). Other examples are epinastine and cetirizine, two P-GP substrates. Kamei et al. (2005) reported that subcutaneous administration of epinastine or cetirizine significantly prolonged the duration of pentobarbital-induced loss of the righting reflex in diabetic mice not normal mice.

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Chapter 10
Roles of Drug Transporters in Blood-Retinal Barrier

Li Liu and Xiaodong Liu

Abstract Blood-retinal barrier (BRB) includes inner BRB (iBRB) and outer BRB (oBRB), which are formed by retinal capillary endothelial (RCEC) cells and by retinal pigment epithelial (RPE) cells in collaboration with Bruch's membrane and the choriocapillaris, respectively. Functions of the BRB are to regulate fluids and molecular movement between the ocular vascular beds and retinal tissues and to prevent leakage of macromolecules and other potentially harmful agents into the retina, keeping the microenvironment of the retina and retinal neurons. These functions are mainly attributed to absent fenestrations of RCECs, tight junctions, expression of a great diversity of transporters, and coverage of pericytes and glial cells. BRB existence also becomes a reason that systemic administration for some drugs is not suitable for the treatment of retinal diseases. Some diseases (such as diabetes and ischemia-reperfusion) impair BRB function via altering tight junctions, RCEC death, and transporter expression. This chapter will illustrate function of BRB, expressions and functions of these transporters, and their clinical significances.

Keywords Blood-retinal barrier · Transporters · Retinal capillary endothelium · Retinal pigment epithelium · Diabetic retinopathy

10.1 General Introduction

The retina has a unique position in that blood-retinal barrier (BRB) separates the retina from the circulating blood. The BRB regulates fluids and molecular movement between the ocular vascular beds and retinal tissues, prevents leakage of macromolecules and other potentially harmful agents into the retina, and keeps the microenvironment of the retina and retinal neurons. BBB existence is also a reason that

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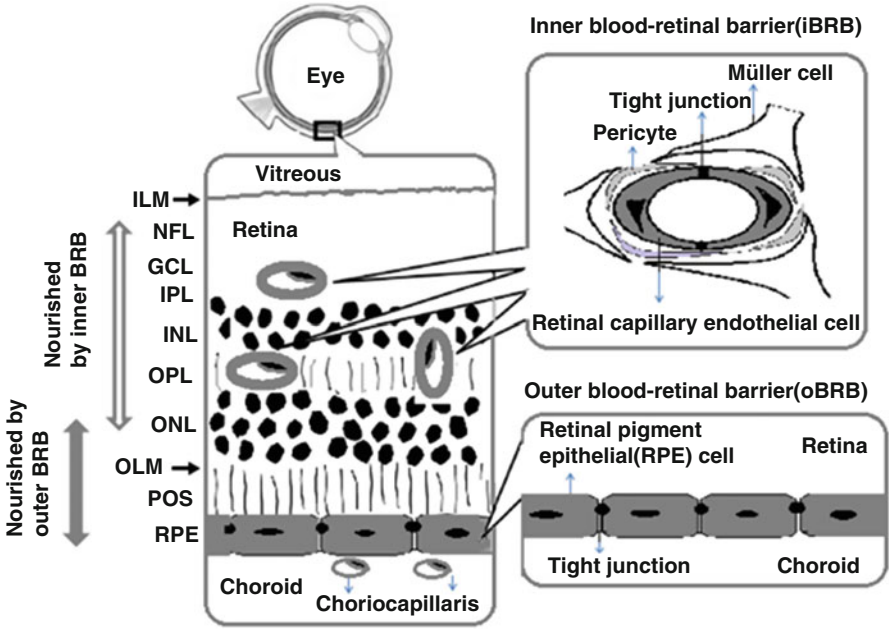

Fig. 10.1 Schematic diagram of blood-retinal barrier (BRB). Symbol: *ILM* inner limiting "membrane," *NFL* nerve fiber layer, *GCL* ganglion layer, *IPL* inner plexiform, *INL* inner nuclear layer, *OPL* outer plexiform, *ONL* outer nuclear layer, *OLM* outer limiting "membrane," *POS* photoreceptor outer segments

systemic drug administration is not suitable for the treatment of retinal diseases. The BRB includes inner BRB (iBRB) and outer BRB (oBRB), which are formed by retinal capillary endothelial cells (RCECs) and by retinal pigment epithelial (RPE) cells in collaboration with Bruch's membrane and the choriocapillaris, respectively (Fig. 10.1). It is well known that two third of the human retina is nourished by retinal capillaries via the iBRB and the remainder is attributed to choriocapillaris via the oBRB (Hosoya and Tomi 2005; Kur et al. 2012).

The paracellular and transcellular transport across BRB are generally involved in the following five different mechanisms (Fig. 10.2) (Rizzolo et al. 2011):

1. Paracellular diffusion: Paracellular diffusion is mainly regulated by the tight junction. Tight junctions, boundaries between the apical and basolateral plasma membrane domains, are considered to be essential for the integrity of tissue barrier and the maintenance of cell polarity, which restrict paracellular movement of fluids and molecules between the blood and retina.
2. Facilitated diffusion: Transporters expressed in the plasma membrane allow the passage of preferred solutes across the monolayer along with a concentration gradient. An example is glucose transport via glucose transporter 1 (GLUT1).

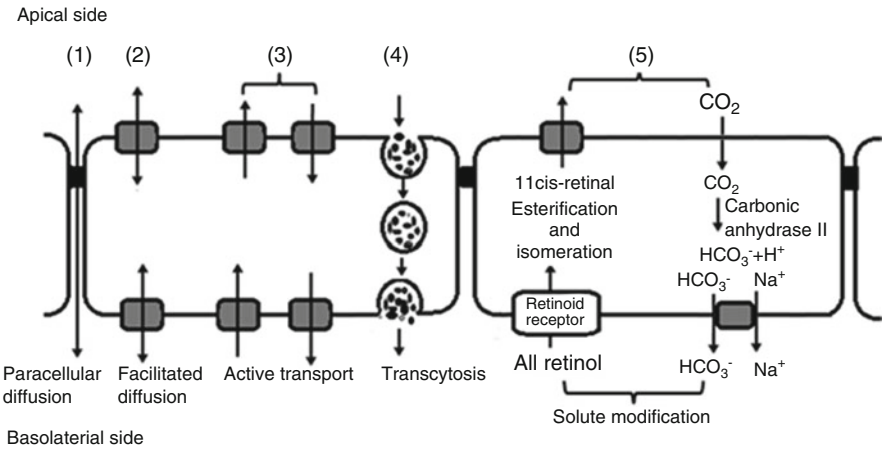

Fig. 10.2 Mechanisms for the transepithelial transport of solutes in the BRB

3. Active transport: Transporters expressed in the plasma membrane consume ATP to move solutes against a concentration gradient or establish electrochemical gradients that drive vectorial transport through antiporters and cotransporters.
4. Transcytosis: Vesicles can invaginate and bud from the apical or basal membrane, traverse the cell, and fuse with the opposite membrane to release their contents on the opposite side of the cell. Normal BRB lacks transcytosis, which become a reason limiting transcellular passage (Chow and Gu 2017).
5. Solute modification: During transport, solutes can be degraded or transformed into something else. For example, in RPE, retinol enters the basal side of the RPE by receptor-mediated endocytosis and is delivered to microsomes, where retinol is transformed into cis-retinal. The cis-retinal transports across the monolayer and is endocytosed by photoreceptors and bound to opsin. Another example is CO_2. CO_2 is converted to HCO_3^- as it is transported from the apical to the basal side of the monolayer.

10.1.1 The Inner Blood-Retinal Barrier (iBRB) and Outer Blood-Retinal Barrier (oBRB)

The iBRB is structurally similar to the blood-brain barrier (BBB). The RCECs connected by tight junctions are covered with pericytes and glial cells (Muller cells or astrocytes) (Cunha-Vaz et al. 2011). The iBRB is formed by the inner or outer capillary beds. The inner capillary bed lies in the ganglion nerve cell layer, and the iBRB function is induced by astrocytes. The outer capillary bed lies in the inner and outer plexiform layers, where function of BRB is regulated by Müller cells (Rizzolo et al. 2011).

The oBRB is established by RPE cells connected by tight junctions. RPE is a monolayer of pigmented cells situated between the neuroretina and the choroids. The apical membrane of RPE exhibiting long microvilli faces the light-sensitive outer segments of the photoreceptors cells, while its basolateral membrane faces the Bruch's membrane, which separates the neural retina from the fenestrated endothelium of the choriocapillaris. It is different from the epithelium of the choroid plexus and other transporting epithelia that the apical membrane of RPE cells abuts a solid tissue rather than a lumen. Moreover, the transepithelial electrical resistance of RPE shows large species differences ranging from 135 to 600 $\Omega \times cm^2$ (Rizzolo et al. 2011).

The main functions of the RPE (Kay et al. 2013; Simó et al. 2010; Willermain et al. 2014a) are to (1) transport nutrients, ions, and water or waste products; (2) absorb light and protect against photooxidation; (3) reisomerize all-*trans*-retinal into 11-*cis*-retinal, which is a key element of the visual cycle; (4) phagocyte shed photoreceptor membranes; (5) release K^+ into the subretinal space to maintain constant excitability of the photoreceptors; (6) secrete growth factors such as pigmented epithelium-derived factor (PEDF) at the apical side and vascular endothelial growth factor (VEGF) at the basolateral side for the structural integrity of the retina; and (7) maintain the immune privilege of the eye due to its outer BRB function but also by interfering with signaling pathways coordinating the immune system.

10.1.2 Tight Junctions in the BRB

Tight junctions consist of specialized proteins such as occludins, claudins, and zonula occludens, which play an important role in maintaining the barrier function via regulating the transport of solutes and molecules across RCEC or RPE cell layers. Both the iBRB and the oBRB cells have tight junctions, but they differ in their organization and composition. Tight junctions of the oBRB are more concentrated at the apical side of the cell, whereas tight junctions of the iBRB are more dispersed between adherens junctions and gap junctions. The adherens junctions consist of vascular endothelial-cadherin and its associated proteins, such as catenins and plakoglobin, both of which are linked to the cytoskeleton (Dejana et al. 2008). The cell–cell contacts also contain additional adhesion molecules, such as platelet endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule 2 (ICAM2), endoglin, and other clusters (Dejana et al. 2008).

10.1.3 Astrocytes, Müller Cells, and Pericytes

Astrocytes, Müller cells, and pericytes, closely connected to blood vessels in the retina, are considered to influence activities of the BRB via secreting regulatory signal molecules such as glial cell line-derived neurotrophic factor (GDNF), transforming growth factor beta 1 (TGF-β1), and VEGF (Igarashi et al. 2000;

Abukawa et al. 2009; Le 2017; Wisniewska-Kruk et al. 2012). In immortalized rat retinal capillary endothelial (TR-iBRB2), an in vitro model cell line of the iBRB, it was found that incubation with conditioned medium of Müller (TR-MUL5) cells increased alkaline phosphatase activity, induced expression of plasminogen activator inhibitor 1 (PAI-1) gene, and suppressed expression of an inhibitor of DNA binding 2 gene. TGF-β1, secreted from TR-MUL5 cells, showed similar effects, indicating that paracrine interactions occur between TR-iBRB2 and TR-MUL5 cells via secreting TGF-β1 (Abukawa et al. 2009). Müller cells are also involved in the regulation of retinal iron homeostasis. Loss of Müller cells was reported to break down the BRB and increase iron levels throughout the neurosensory retina of mice (Baumann et al. 2017). Müller cell loss or dysfunction often occurs in patients with macular telangiectasia type 2 (MacTel2) and diabetic retinopathy, which may explain the clinical findings that a patient with MacTel2 and a patient with diabetic retinopathy had the increased iron levels in RPE or neurosensory retina (Baumann et al. 2017). TGF-β was reported to impair function of BRB via simulating metalloproteinases (MMPs) from Müller cells, later degrading tight junction protein occludin (Behzadian et al. 2001). Moreover, interactions between endothelial cells and pericytes or astrocytes also increase the BRB properties through enhancing expression of tight junctions (Kim et al. 2009).

The pericytes play important roles in regulating vascular tone, secreting extracellular material, and being phagocytic. The frequency of pericyte coverage on human retinal capillaries was reported to be high up to 94.5%, substantially greater than that of human choriocapillaris (11%) (Chan-Ling et al. 2011), demonstrating that retinal microvasculature characterizes a uniquely high density of pericytes. The communication between pericytes and endothelial cells is mediated by diverse molecules such as angiopoietin, TGF-β1, platelet-derived growth factor-β (PDGF-β), and sphingosine-1-phosphate. In endothelial cells, genetic deletion of PDGF-β (Park et al. 2017) or blocking PDGF receptor beta using antibody (Ogura et al. 2017) could lead to severe vascular impairments such as vascular engorgement, leakage, severe hemorrhage, retinal detachment, and severely impaired pericyte coverage of the vessels. Moreover, pericyte loss also promotes pathologic angiogenesis. In consistence, pericyte dropout or loss in the retina is considered to be one of the earliest pathological changes in diabetic retinopathy. Moreover, hyperglycemia, advanced glycation end products, basement membrane thickening, and hypertension trigger pericyte apoptosis and dropout, all of which become reasons leading to diabetic retinopathy (Eshaq et al. 2017).

10.2 Major Drug Transporters in the Retina

Several transporters, including the solute carrier (SLC) family and the ATP-binding cassette (ABC) family have been identified in the retina (Fig. 10.3). The SLC transporters utilize facilitated diffusion, or they couple an ion or electrochemical

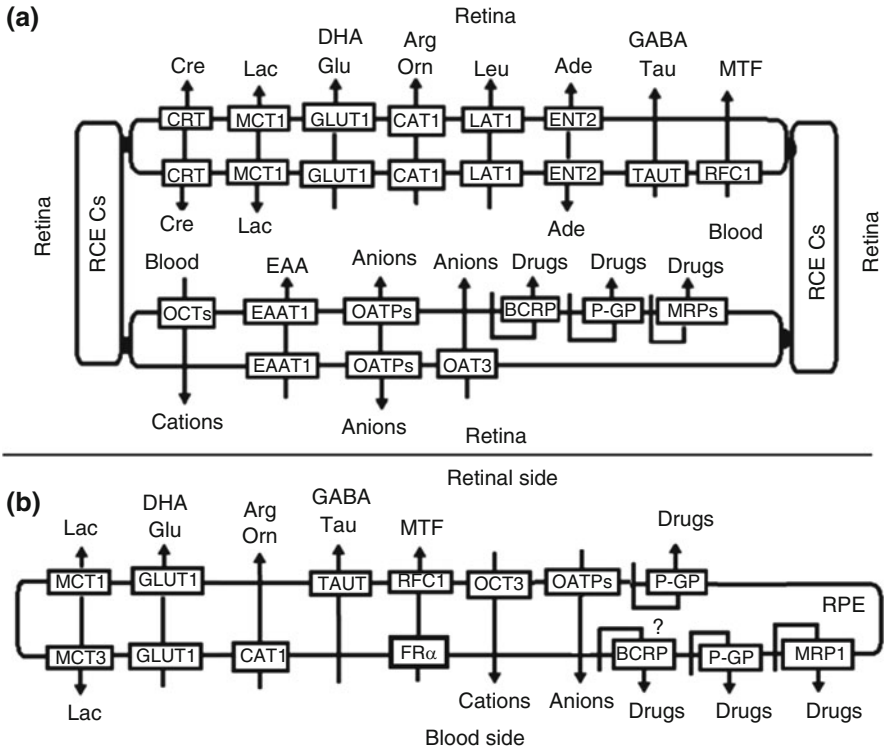

Fig. 10.3 Hypothetical localization and physiological function of several transporters in the iBRB (**a**) and the oBRB (**b**). *Ade* adenosine, *Arg* L-arginine, *Cre* creatine, *DHA* dehydroascorbic acid, *EAA* excitatory amino acid, *GABA* gamma-aminobutyric acid, *Glu* glucose, *Lac* lactate, *Leu* L-leucine, *MTF* methyltetrahydrofolate, *Orn* L-ornithine, *RCECs* retinal capillary endothelial cells, *RPE* retinal pigment epithelial (RPE) cells, *Tau* taurine

gradient to transfer their substrates across the cell membrane. ABC transporters, on the other hand, use ATP as the energy source to drive the transport.

In the retina, neuronal cells, including photoreceptor cells, require a large amount of metabolic energy for phototransduction and neurotransduction metabolic substrates, such as D-glucose, amino acids, vitamins, and nucleosides. These compounds are hydrophilic, and their transport is often mediated by influx transporters, belonging to SLC family. The identified influx transporters in the retina include glucose transporter 1 (GLUT1), Na^+-dependent multivitamin transporter (SMVT), taurine transporter (TAUT), cationic amino acid transporter 1 (CAT1), excitatory amino acid transporter 1 (EAAT1), L-type amino acid transporter 1 (LAT1), creatine transporter (CRT), nucleoside transporters, and monocarboxylate transporters (MCTs). A series of influx transporters for drugs such as organic cation transporters (OCTs), organic anion transporting polypeptides (OATPs), and organic anion transporters (OATs) have been also identified in the retina.

10.2.1 Influx Transporters

10.2.1.1 Glucose Transporter 1 (GLUT1/SLC2A1)

D-glucose is the main energy source for the retina, whose transport from the blood to the retina is mainly mediated by GLUT1 (Tomi and Hosoya 2004). GLUT1 is mainly localized at both luminal and abluminal membranes of the iBRB and the oBRB, although the expression of GLUT1 at abluminal membrane of the iBRB is approximately two- to threefold greater than that at its luminal membrane (Fernandes et al. 2003). The asymmetrical distribution of GLUT1 at the iBRB suggests that D-glucose transport is limited at the blood-to-luminal rather than the abluminal-to-interstitial interface. GLUT1 is also expressed in Müller cells (Hosoya et al. 2008a). In addition to hexoses, GLUT1 also transports dehydroascorbic acid from blood to the retina, where dehydroascorbic acid is converted to ascorbic acid. The uptake of dehydroascorbic acid by GLUT1 may be completely inhibited under diabetic conditions due to high glucose concentration (Minamizono et al. 2006).

10.2.1.2 Taurine Transporter (TAUT/SLC6A6)

Taurine, functioning as osmolyte and antioxidant, is the most abundant free amino acid in the retina, accounting for more than 50% of the free amino acid content in the rat retina, which is considered to be essential for maintenance of retinal structure. Taurine transport across BRB is mainly mediated by TAUT (Tomi et al. 2007). TAUT, an Na^+- and Cl^--dependent transporter, is mainly expressed in apical membrane of RPE, ganglion cells, Müller cells (El-Sherbeny et al. 2004), and RCECs (Tomi et al. 2007, 2008). Some TAUT inhibitors such as β-alanine and hypotaurine may inhibit taurine transport across BRB. TAUT also mediates transport of β-alanine and gamma-aminobutyric acid (GABA) (Tomi et al. 2008; Usui et al. 2013). Hyperosmolar conditions could stimulate activity of TAUT, leading to increase in V_{max} of taurine without affecting K_m (El-Sherbeny et al. 2004; Yahara et al. 2010). Both in vivo and in vitro data demonstrated that taurine itself enhanced expression of retinal TAUT (Zeng et al. 2010). High glucose was reported to downregulate expression of retinal TAUT (Lee and Kang 2013; Stevens et al. 1999), which was attenuated by taurine treatment (Zeng et al. 2010). In consistence with this issue, 8-week and 12-week diabetic rats demonstrated significantly lower levels of retinal TAUT, and taurine treatment completely reversed the decreased expression of retinal TAUT by diabetes (Zeng et al. 2010). Similarly, TAUT deficiency was reported to lead to severe retinal degeneration in mice (Heller-Stilb et al. 2002). All these results demonstrated important roles of retinal TAUT in normal retinal development.

10.2.1.3 Cationic Amino Acid Transporter 1 (CAT1/SLC7A1)

L-arginine is the precursor molecule for the synthesis of nitric oxide (NO) by nitric oxide synthase (NOs). L-arginine transport from the blood to the retina across BRB is mediated by CATs. CAT1 is highly expressed at both luminal and abluminal membrane of RCECs, TR-iBRB2 cells, the basal membrane of PRE (Kubo et al. 2015; Tomi et al. 2009), and immortalized rat retinal pericyte cell line (TR-rPCT1 cells) (Zakoji et al. 2015). QT-PCR analysis showed that levels of CAT1 mRNA in TR-iBRB2 and the isolated rat RCECs were 25.9- and 796-fold greater than that of CAT3, respectively. CAT1-specific small interfering RNA, L-arginine, and L-lysine inhibited [^{3}H]L-arginine uptake in TR-iBRB2 cells (Tomi et al. 2009). Moreover, high glucose exposure significantly inhibited L-arginine transport in TR-iBRB, and simvastatin might reverse high glucose-induced-alterations via increasing expression of endothelial NOS mRNA and NO production (Tun and Kang 2017).

L-ornithine is a cationic amino acid produced in the urea cycle and ingested from the diet. Clinical trials have suggested the beneficial effects of L-ornithine in the body, but long-term treatment or high concentrations of L-ornithine in the blood induce retinal toxicity, forming gyrate atrophy of the choroid and retina (Hayasaka et al. 2011), an autosomal recessive disease due to the genetic defect of ornithine aminotransferase. Both in vivo and in vitro data demonstrated that CAT1 mediates transport of [^{3}H]L-ornithine across the BRB (Kubo et al. 2015). In RPE cells, it was reported that the basal-to-cell uptake of [^{3}H]L-ornithine was greater than that of the apical-to-cell uptake, and the basal-to-cell transport was inhibited by L-ornithine, suggesting the involvement of CAT1 in the blood-to-cell transport of L-ornithine across the basal membrane of the oBRB (Kubo et al. 2015). Moreover, in human telomerase reverse transcriptase-RPE cells, CAT1 siRNA decreased both L-[^{14}C] ornithine uptake and L-ornithine cytotoxicity (Kaneko et al. 2007). All these results indicate that reduction of the ornithine transport via inhibiting CAT1 may be a new target for treatment of gyrate atrophy.

10.2.1.4 L-Type Amino Acid Transporter 1 (LAT1/SLC7A5)

L-type amino acid transporters (LATs) prefer branched-chain and aromatic amino acids. LAT1 (SLC7A5) and LAT2 (SLC7A8) mRNAs were reported to be expressed in cultured human RPE cell line (ARPE-19 cells), but the level of LAT1 mRNA was 42-fold higher than that of LAT2 (Yamamoto et al. 2010). LAT1, an Na^+-independent transporter, was also identified in TR-iBRB2, isolated rat RCECs, and primary cultured human RCECs (Tomi et al. 2005; Usui et al. 2013; Yamamoto et al. 2010), mediating blood-to-retina transport of large neutral amino acids including L-leucine (Tomi et al. 2005), L-histidine (Usui et al. 2013), and L-phenylalanine (Atluri et al. 2008). Some drugs such as L-dopa, melphalan, alpha-methyldopa, and gabapentin are substrates of LAT1 and LAT2 (del Amo et al. 2008), indicating the roles of LAT1 in retinal disposition of these drugs.

10.2.1.5 Creatine Transporter (CRT/SLC6As)

Creatine and phosphocreatine are required to maintain ATP needed for normal retinal function and development. Creatine is transported from the blood to the retina against the creatine concentration gradient via creatine transporter (CRT), an Na^+- and Cl^--dependent transporter. The creatine transport from the blood to the retina is considered to be a major pathway for supplying creatine to the retina although local creatine is preferentially synthesized in the glial cells (Tachikawa et al. 2007; Nakashima et al. 2005a). In the inner retina, CRT is expressed in cells of intense metabolic activity, such as photoreceptors and selected cells, but not in glial cells (de Souza et al. 2012). CRT is also expressed at both the luminal and abluminal membranes of the iBRB (Nakashima et al. 2004). CRT expressed in the luminal membrane would mediate creatine supply to the retina, and CRT in the abluminal membrane may be involved in the metabolite uptake of creatine (Nakashima et al. 2004).

10.2.1.6 Monocarboxylate Transporters (MCTs/SLC16As)

The retina produces more L-lactic acid aerobically than any other tissues. In addition to D-glucose, L-lactic acid appears to be required as an energy source in photoreceptors. Transports of L-lactic acid and other monocarboxylates (such as pyruvate and the ketone bodies across cellular membranes) are facilitated by specific monocarboxylate transporters (MCTs). Four MCTs (including MCT1, MCT2, MCT3, and MCT4, encoded by SLC16A1, SLC16A7, SLC16A8, and SLC16A3, respectively) have been identified in the retina (Bergersen et al. 1999; Gerhart et al. 1999; Philp et al. 1998). MCT1 is detected on four retinal cell types: RPE, photoreceptor cells, Müller cells, and endothelial cells (Gerhart et al. 1999). MCT1 is mainly located in the apical membrane of RPE and in both the luminal and abluminal plasma membranes of RCECs (Bergersen et al. 1999; Gerhart et al. 1999). MCT2 is only found to be abundantly expressed on the inner (basal) plasma membrane of Müller cells and by glial cell processes surrounding retinal microvessels (Gerhart et al. 1999). High expression of MCT4 is only detected in the RPE of younger animals, but RPE of adult animals only show very weak expression (Bergersen et al. 1999). MCT3 is preferentially expressed in the basolateral membrane of the RPE (Philp et al. 1998, 2001), forming a heteromeric complex with the accessory protein CD147 (Philp et al. 2001). The absence of *Mct3* was reported to impair expression of CD147 from the basolateral but not apical RPE of mice. Moreover, the amount of L-lactate in retinal of $Mct3^{-/-}$ mice was approximately four times higher than that from the wild-type retinas, accompanied by decreases in the magnitude of the light suppressible photoreceptor current (Daniele et al. 2008). These results demonstrate the pivotal roles of MCT3 in regulating the ionic composition of the outer retina.

MCTs also accept monocarboxylic acid drugs such as moxifloxacin (Barot et al. 2014) and nicotinate (Tachikawa et al. 2011) as their substrates, indicating that

MCT-mediated transport at BRB is a possible route for delivery of monocarboxylic acid drugs to the retina (Hosoya et al. 2001).

10.2.1.7 Nucleoside Transporters

Adenosine is an important intercellular signaling molecule, showing a number of roles in retinal neurotransmission. Most of the adenosine in the retinal interstitial fluid originates from the catabolism of adenosine monophosphate, which is localized in the innermost process of Müller cells. Thus, almost all of the retinal adenosine is distributed in the neighborhood of the innermost process of Müller cells in the ganglion cell layer, inner plexiform layer, and inner nuclear layer. Adenosine in the blood may penetrate the iBRB via adenosine transport systems. Four transporters including two equilibrative nucleoside transporters (ENT1/(ENT2, SLC29A1/SLC29A2) and two concentrative nucleoside transporters (CNT1/CNT2, SLC28A1/SLC28A2) have been identified in the rat retina, although the expression of ENT2 mRNA was reported to be 5.5-fold greater than that of ENT1 mRNA. Among the four transporters, only CNT1 was not detected in TR-iBRB. Adenosine uptake in TR-iBRB2 cells is predominantly mediated by ENT2, which is strongly inhibited by adenosine, inosine, uridine, and thymidine, but neither nitrobenzylmercaptopurine riboside (NBMRP) nor dipyridamole, characterizing NBMPR- and dipyridamole-insensitive transport of adenosine. An in vivo study suggested that [^{3}H]adenosine transport from the blood to the retina was also significantly inhibited by adenosine and thymidine, demonstrating that ENT2 most likely mediates adenosine transport at the iBRB (Nagase et al. 2006). ENT2 is also expressed in Müller cells, contributing to transport of adenosine and its metabolite hypoxanthine. ENT2 also mediates elimination of hypoxanthine from the retina (Akanuma et al. 2013a). In addition, ENT2 accepts some antiviral or anticancer nucleoside drugs, such as 3′-azido-3′-deoxythymidine, 2′3′-dideoxycytidine, 2′ 3-′-dideoxyinosine, cladribine, cytarabine, fludarabine, gemcitabine, and capecitabine, as preferred substrates (Baldwin et al. 2004; Yao et al. 2001), indicating that ENT2 at the BRB could be a potential route for delivering nucleoside drugs from the circulating blood to the retina.

10.2.1.8 Folate Transport Proteins

Folates, water-soluble vitamins, play an essential role as cofactors for one-carbon metabolism in cells. Most of the folate in the plasma is in the reduced form, methyltetrahydrofolate. Folate transport from blood to the retina across BRB is mediated by some specific transport process. Three transport proteins, folate receptor-α (FRα), reduced folate carrier 1 (RFC1/SLC19A1), and proton-coupled folate transporter (PCFT/SLC46A1), have been described for folate uptake. FRα, a receptor for folate, is expressed in basolateral membrane of PRE and in retinal Müller cells, mediating the influx of its ligand into cells via receptor-mediated endocytosis

(Bozard et al. 2010; Chancy et al. 2000). RFC1, a pH-sensitive transporter, is involved in folate^{-}/OH^{-}exchange. RFC1 is highly expressed in TR-iBRB2 cells, isolated rat RCECs, normal mouse RPE, and in cultured human RPE cells (Chancy et al. 2000; Hosoya et al. 2008b). Although both RFC1 and PCFT mRNA are expressed in TR-iBRB2 cells and isolated rat RCECs, the expression level of RFC1 mRNA was reported to be 83- and 49-fold greater than that of PCFT, respectively, indicating that RFC1 probably predominates at the iBRB. In TR-iBRB2 cells, transports of methotrexate, formyltetrahydrofolate and methyltetrahydrofolate are meidiated by RFC1 (Hosoya et al. 2008b). RFC1 is also expressed in RPE cells. Importantly, FRα and RFC1 are localized in the basolateral and apical membrane of the RPE, respectively, demonstrating that the two proteins work in a concerted manner to operate the vectorial transfer of folate across RPE from choroidal blood to the retina. Diabetes might downregulate expression and activity of RFC1 in mouse PRE. In ARPE-19 cells, it was reported that 6 h exposure of high glucose (45 mM) led to decreases in methyltetrahydrofolate uptake by 35%, which was consistent with decreases in levels of RFC1 mRNA and protein (Naggar et al. 2002). PCFT mRNA was detected in all tested retinal cells including primary cultures of ganglion, Müller, and RPE cells of the mouse retina and their cell lines (Umapathy et al. 2007), mediating H^{+}-coupled transport of folate. In retinal Müller cells, PCFT and FRα are expressed and colocalized in the endosomal compartment, where the two proteins may work coordinately to mediate folate uptake (Bozard et al. 2010).

10.2.1.9 Organic Anion-Transporting Polypeptides (OATPs)

Several OATPs (OATPs for human and Oatps for animal), such as Oatp1a4, Oatp1c1, Oatp4a1, and Oatp1a5, have been detected in rat retina. Oatp1a4 and Oatp1c1 mRNA are predominantly expressed in isolated rat RCECs (Tomi and Hosoya 2004). Oatp1a4 proteins are detected on both the abluminal and luminal membrane of rat RCECs and RPE, but Oatp1a4 protein is preferentially localized on the abluminal membrane of the RCECs and the apical membrane of rat RPE (Akanuma et al. 2013b; Ito et al. 2002). Oatp1c1 protein is expressed in both the abluminal and luminal membrane of the RCECs and is preferentially expressed in the basolateral membrane of rat RPE (Akanuma et al. 2013b; Ito et al. 2002). Oatp1a5 protein is predominantly localized in optic nerve fibers, not in RPE (Gao et al. 2002; Ito et al. 2002). Expressions of Oatp1a4 and Oatp1c1 on two side membranes of BRB indicate their contributions to the transcellular transport of amphipathic organic anions including digoxin and [^{3}H]estradiol 17-β glucuronide (E17βG) across the BRB in both the blood-to-retina and retina-to-blood directions. It was found that elimination rate constant of [^{3}H]- E17βG from the vitreous humor of rats was 1.9-fold greater than that of [^{14}C]D-mannitol. The efflux transport of E17βG from rat retina was significantly inhibited by organic anions probenecid, sulfobromophthalein, digoxin, and dehydroepiandrosterone sulfate (Katayama et al. 2006). Oatp4a1 is also expressed in the RPE, inner and outer nuclear layers,

ganglion cell layer, and nerve fiber layer of rat eyes. In the cultured rat RPE cells, it was found that uptake of triiodothyronine, a known substrate of Oatp4a1, was significantly inhibited by OATP inhibitor sulfobromophthalein, indicating roles of OATPs in the transport of thyroid hormone in the retina (Ito et al. 2003).

Human OATP1A2 is expressed in photoreceptor bodies, somas of amacrine cells and RPE, and mediates the cellular uptake of all-trans-retinol (Chan et al. 2015; Gao et al. 2015), inferring roles of OATP1A2 in canonical visual cycle. The OATP1A2-mediated uptake of all-trans-retinol may be inhibited by chloroquine and hydroxychloroquine (Xu et al. 2016), which may provide novel insights into retinal dysfunction induced by certain drugs. For instance, digoxin and antimalarial drugs (chloroquine and hydroxychloroquine) have induced retinopathy (Kinoshita et al. 2014; Weleber and Shults 1981; Yaylali et al. 2013), which may be partly contributed to decrease in all-trans-retinol uptake into the retinal cells via inhibiting retinal OATP1A2 function, resulting in dysfunction of the canonical visual cycle and toxic accumulation of retinoids. Moreover, human OATP1A2 and OATP2B1 are abundantly expressed in retina amacrine neurons containing substance P and vasoactive intestinal peptide. The two peptides are also substrates of OATP1A2 and OATP2B1, demonstrating roles of OATP1A2 and OATP2B1 in reuptake of these neuropeptides released from retinal neurons and in the homeostasis of neuropeptides (Gao et al. 2015).

10.2.1.10 Organic Cation Transporters

Organic cation transporter 3 (Oct3) is detected in mouse RPE and in several cell types of the neural retina, including photoreceptor, ganglion, amacrine, and horizontal cells, where Oct3 participates in the clearance of dopamine, histamine, and neurotoxin 1-methyl-4-phenyl pyridinium (MPP^+) from the subretinal space (Rajan et al. 2000). In cultured ARPE-19 cells, it was found that uptake of typical OCT3 substrate MPP^+ was completely inhibited by several cationic drugs and monoamine neurotransmitters (dopamine and histamine) (Rajan et al. 2000), although an organic cation transporter, functionally similar to plasma membrane monoamine transporter (SLC29A4), was reported to mediate retina-to-blood transport of MPP^+ at BRB (Kubo et al. 2017a).

L-carnitine is essential for the translocation of acylcarnitine esters into mitochondria for β-oxidation of long-chain fatty acids and ATP generation. L-carnitine transport in the retina is mainly mediated by organic cation/carnitine transporter 1/organic cation/carnitine transporter 2 (OCTN1/OCTN2 for human and Octn1/Octn2 for animal). QT-PCR analysis showed that the expressions of Octn2 mRNA in TR-iBRB2 and isolated rat RCECs were 27.3- and 45.9-fold greater than Octn1 mRNA, respectively, indicating that Octn2 predominantly accounts for the transport of acetyl-L-carnitine from the blood to the retina across the iBRB (Tachikawa et al. 2010). In consistence, acetyl-L-[^{3}H]carnitine uptake in rat retina was significantly suppressed by L-carnitine and acetyl-L-carnitine. In TR-iBRB2 cells, OCTN substrates and inhibitors (as L-carnitine, acetyl-L-carnitine, tetraethylammonium,

quinidine, and betaine) remarkably decreased uptake of L-[^{3}H]carnitine and acetyl-L-[^{3}H]carnitine (Tachikawa et al. 2010), further confirming roles of retina OCTNs in transport of L-carnitine.

Several novel organic cation transporters have been identified. Transport of the cationic drugs clonidine and diphenhydramine at the mouse BRB is mainly attributed to a carrier-mediated system. The transporter for transporting clonidine is an Na^+-independent proton-antiporter and insensitive to the *trans*membrane potential. The transporter also transports other cationic drugs such as nicotine, tramadol, diphenhydramine, cocaine, verapamil, methadone, and oxycodone (Chapy et al. 2015). Transport of [^{3}H]verapamil across the iBRB is also mediated by a novel organic cation transporter. In vivo data demonstrated that influx transport of verapamil across the BRB was about fivefold higher than that across the BBB. Verapamil (3 mM) and quinidine (10 mM), not pyrilamine (3 mM), slightly increased the retinal uptake of [^{3}H]verapamil. However, these compounds markedly increased brain uptake of [^{3}H]verapamil. Moreover, pyrilamine (40 mM) significantly reduced the retinal uptake index to 72.9% but not for the brain uptake index. These in vivo results clearly demonstrate that differently from the BBB, transport of verapamil across the BRB is mediated by both influx transporters and efflux transporters (Kubo et al. 2013a). Importantly, in P-GP-deficient rats, P-GP inhibitors (vinblastine and verapamil) inhibited verapamil uptake by the retina but not the brain, which conformed that [^{3}H]verapamil is permeated across the BRB via influx transporters (Fujii et al. 2014). In human RPE cell lines (RPE/Hu and ARPE-19), it was found that verapamil uptake is active, pH-dependent, and independent of the membrane potential (Han et al. 2001), but in TR-iBRB2 cells, verapamil uptake was independent of pH (Kubo et al. 2013a), indicating that it has characteristics of influx transporter for verapamil in the iBRB was different from that in the oBRB. The verapamil uptake was inhibited by metabolic inhibitors, quinidine, pyrilamine, diphenhydramine, diltiazem, timolol, propranolol, and L-carnitine, but not by other known OCT/OCTN2 substrates nor inhibitors (such as tetraethylammonium, cimetidine, decynium-22, and MPP^+) (Han et al. 2001; Kubo et al. 2013a). Similarly, [^{3}H]pyrilamine uptake in TR-iBRB2 cells was also inhibited by verapamil and some cation compounds (such as desipramine, imipramine, propranolol, memantine, quinidine, and nipradilol), not tetraethylammonium, serotonin, choline, or choline. However, the transporter mediating pyrilamine uptake seemed not to be identical to verapamil transport system, because they showed different sensitivity to pH and L-carnitine. Moreover, kinetic analysis indicated that verapamil had no competitive effect on the pyrilamine uptake although verapamil uptake exhibited a competitive-like inhibition (Kubo et al. 2013a).

Transport of propranolol across iBRB is mediated by an influx carrier and was reduced by several organic cations (Kubo et al. 2013b). Propranolol uptake in TR-iBRB2 cells was also inhibited by some organic cations (such as pyrilamine, verapamil, imipramine) but not substrates nor inhibitors of OCTs (MPP^+, tetraethylammonium, and cimetidine). The propranolol transport is pH dependent, Na^+ independent, and L-carnitine insensitive, which is different from verapamil transport system (Kubo et al. 2013a, b). Blood-to-retina transport of nicotine across the iBRB is driven by an outwardly directed H^+ gradient, which was stimulated by

an outwardly directed H^+ gradient and significantly inhibited by organic cations (pyrilamine and verapamil) but not OCT inhibitors (Kubo et al. 2014; Tega et al. 2015). These findings that substrate specificity of the cationic drug transport at the BRB is different from those of well-characterized organic cation transporters OCTs, OCTNs, and MATEs, suggesting the involvement of novel organic cation transporters in the influx transport of these cationic drugs across the BRB (Chapy et al. 2015; Kubo et al. 2013a, b, 2014).

10.2.1.11 Other Transporters

Organic anion transporter 3 (OAT3) is expressed in RCECs and TR-iBRB cells. Rat Oat3 is possibly located at the abluminal membrane of the RCECs, where it effluxes its substrates such as *p*-aminohippuric acid, benzylpenicillin, and 6-mercaptopurine from the vitreous humor/retina to the blood across the iBRB, limiting the retinal distribution of these substrates (Hosoya et al. 2009). Unlikely BBB, androgen receptor ligand dihydrotestosterone did not affect expression of Oat3 mRNA in TR-iBRB cells (Ohtsuki et al. 2005). Anion transporter inhibitor probenecid also inhibited transport of digoxin across BRB of rats, leading to significant increases in uptake retina index by 1.6-fold of control, indicating roles of OATs in efflux of digoxin across BRB (Toda et al. 2011).

Excitatory acid transporter 1 (EAAT1), an Na^+-dependent high-affinity L-glutamate transporter, is localized on the abluminal membrane of the RCECs and mediates elimination of L-glutamate, a neuroexcitatory neurotransmitter, from the retina across the iBRB (Sakurai et al. 2015). Multivitamin transporter (SMVT/SLC5A6), an Na^+-dependent transporter, was detected in TR-iBRB2 cells and isolated rat RCECs, mediating the uptake of vitamins and some essential cofactors such as biotin, pantothenic acid, and lipoic acid (Quick and Shi 2015). In vivo, [^{3}H]biotin uptake by the rat retina was significantly inhibited by biotin and pantothenic acid. [^{3}H]biotin uptake in TR-iBRB2 cells was also significantly inhibited by biotin, pantothenic acid, lipoic acid, and desthiobiotin (Ohkura et al. 2010). SMVT mRNA and protein were also detected in human RPE cells. Hypoxia induced expression and function of SMVT in human RPE cells, indicating that hypoxia may alter disposition of ophthalmic drugs (Vadlapatla et al. 2013). Riboflavin transport across blood-to-retina is mediated by riboflavin transporter (SLC52A/RFVT), an Na^+- and Cl^--independent transporter. In TR-iBRB2 cells, two RFVTs RFVT2 (SLC52A2) and RFVT3 (SLC52A3) were detected (Kubo et al. 2017b).

10.2.2 Efflux Transporters

Efflux transporters mainly belong to ABC family transporters. Several ABC transporters, such as P-glycoprotein (P-GP/*ABCB1*), multidrug resistance proteins

(MRPs/*ABCCs* for human and Mrps/*ABccs* for animal) (Asashima et al. 2006), and breast cancer resistance protein (BCRP/*ABCG2*), were identified in the BRB.

10.2.2.1 P-Glycoprotein (P-GP/ABCB1)

P-GP is mainly expressed at the iBRB. However, reports on P-GP in the oBRB are often contradictory, which may result from different tissues or cells from several species or different detection methods. In mice, it was found that the oBRB showed low or lack expression of P-GP (Chapy et al. 2016). In human fetal RPE and ARPE-19, no P-GP protein was detected by LC-MS/MS (Pelkonen et al. 2017). But, in the oBRB tissue of porcine, P-GP protein was measured to be 2.01 fmol/μg protein by LC-MS/MS, although it was less than that of the iBRB (8.70 fmol/μg protein) (Zhang et al. 2017a). Similarly, among three human RPE cell lines (ARPE-19, D407 and h1RPE), only ARPE-19 cells do not express P-GP. D407 and h1RPE cells express P-GP, but functional activity is demonstrable only in D407 cells (Constable et al. 2006). Expression and function of P-GP were also detected in both cultured human RPE and in D407 cells. Furthermore, P-GP immunoreactivity is predominantly associated with localization to both apical and basolateral cell membranes of human RPE (Kennedy and Mangini 2002). Importantly, expression and function of P-GP are also demonstrated in the mitochondria of D407 cells and upregulated by H_2O_2 (Zhang et al. 2017b). Expression and function of P-GP were also clearly demonstrated in the oBRB tissue of porcine. Permeabilities of verapamil and rhodamine in the retina-to-choroid direction were reported to be higher 5.5- and 2.6-fold than those in the opposite direction. Moreover, cellular calcein accumulation in the presence of verapamil was twice as strong as control (Steuer et al. 2005). PET data showed that P-GP inhibitor tariquidar significantly increased influx rate constant k_1 across the BRB and total retinal distribution volume of (R)-[^{11}C]-verapamil in human subjects, by 1.4-fold and 1.5-fold of baseline. In accordance with this, retinal efflux rate constant k_2 was significantly decreased by 2.8 in the presences of P-GP inhibitor (Bauer et al. 2017).

It is worth noting that the impact of P-GP on BRB permeability to its substrates is greatly lower than that on BBB permeability (Chapy et al. 2016; Fujii et al. 2014). For example, uptake indexes of verapamil, quinidine, and digoxin in the retina of *Abcb1a1*$^{-/-}$ rats were 1.6-, 1.07-, and 3.7-fold of wild-type rats, respectively. But, the brain uptake parameters for verapamil, quinidine, and digoxin were high up to 8.3-, 12.3-, and 14.0-fold of wild-type rats, respectively. Quinidine, verapamil, and digoxin are substrates of P-GP, but P-GP inhibitors only inhibited transport of digoxin in the retina, which was different from the brain. These results indicate that P-GP may play a substantial role in the retinal distribution of digoxin, but not verapamil nor quinidine (Toda et al. 2011), which may partly be attributed to influx transporters for these drugs. Similarly, transport of [^{3}H]-verapamil at BRB of mice was significantly increased by ~1.5-fold following P-GP inhibition using elacridar (5 μM) or valspodar (5 μM) and by 1.3-fold in triple knockout (*Abcb1a/Abcb1b*$^{-/-}$ and *Abcg2*$^{-/-}$) mice compared with control wild-type mice, but extents of these

alterations at the BRB were remarkably lower than those (5.6-fold for elacridar, 8.4-fold for valspodar, and 10.3-fold for triple knockout) at the BBB (Chapy et al. 2016).

10.2.2.2 Breast Cancer Resistance Protein (BCRP/ABCG2)

Likely to P-GP, BCRP is mainly located at the luminal membrane of RCECs (Asashima et al. 2006; Chapy et al. 2016), acting as the efflux transporter for photosensitive toxins and drugs in retinal tissue. In TR-iBRB2 cells, BCRP mediates cellular efflux of phototoxic compounds pheophorbide and protoporphyrin IX, which is inhibited by ABCG2 inhibitor Ko143 (Asashima et al. 2006). BCRP is often co-located with P-GP in the retina. In mice, P-GP and BCRP are uniformly expressed in the physiologically developing retinal vasculature of the neonatal mouse (Tagami et al. 2009). Expressions of P-GP and BCRP mRNA and proteins were also detected in the retinal vascular endothelial cells from the adult mouse retina (Tachikawa et al. 2008; Chapy et al. 2016).

Similarly to P-GP, the importance of BCRP efflux at the retina is less than that at the BBB. Triple knockout (*Abcb1a/Abcb1b*$^{-/-}$*:Abcg2*$^{-/-}$) and coadministration of BCRP inhibitor elacridar significantly increased the mitoxantrone entry rate to the mouse brain, with 3.3-fold increases of control mice, but these increases did not occur at the retina (Chapy et al. 2016).

Several reports have also demonstrated expression of BCRP in the oBRB, although contradictory may be often contradictory. LC-MS/MS analysis demonstrated no expression of BCRP in human fetal RPE and ARPE19 (Pelkonen et al. 2017), but high levels of BCRP protein were still detected in both the iBRR (22.8 fmol/μg protein) and the oBRB (2.76 fmol/μg protein) of pig (Zhang et al. 2017a). Among the tested three human RPE cell lines (ARPE-19, D407, and HRPEpiC) and bovine primary cells, BCRP was only detected in D407 cells (Mannermaa et al. 2009). However, BCRP mRNA was also detected in the neural retina, RPE eyecup, and primary mouse RPE cells. Immunoreactivity showed that the expression of BCRP is almost exclusively restricted to the RPE cell layer, mainly at the basolateral membrane of PRE (Gnana-Prakasam et al. 2011), indicating that the function of BCRP in the oBRB is to efflux of heme from the retina to choroidal blood, showing roles in retinal hemochromatosis. Iron overload downregulated BCRP expression in PRE, whose defective function in RPE may lead to increases in the cellular levels of phototoxin, thus contributing to oxidative stress and enhancing the progression of retinal diseases such as age-related macular degeneration (Gnana-Prakasam et al. 2011).

10.2.2.3 Multidrug Resistance-Associated Proteins (MRPs/ABCCs)

MRPs seem to be mainly expressed in the oBRB, but different tissues or cells from several species or detected methods often show different patterns of MRP expression. Mannermaa et al. (2009) investigated expressions of several MRPs in three

human RPE cell lines (ARPE-19, D407 and HRPEpiC) and bovine primary RPE cells. The results showed that expressions of MRP1, MRP4, and MRP5 proteins were detected in the tested three human RPE cell lines. Unlike MRP1 and MRP4, Mrp5 protein was not detected in bovine primary RPE cells. MRP2 protein was only detected in the D407 cells (Mannermaa et al. 2009). Activities of MRP1 and MRP5 in ARPE-19 cells were also confirmed by the calcein-AM and CDCF efflux tests (Mannermaa et al. 2009), respectively. In PRE of porcine, permeability of known MRP substrate fluorescein in the retina-to-choroid direction across was reported to be higher 11.3-folds than that in opposite direction. The transport of fluorescein in the retina-to-choroid direction was blocked by probenecid, with the result that permeability was equalized in both directions, which was in line with expression of Mrps (Steuer et al. 2005).

LC-MS/LC-MS analysis demonstrated high expression of MRP1 in primary human PER cells, ARPE-19 cells, and oBRB tissue of porcine. High levels of MRP5 protein were only detected in primary human PER cells but not ARPE-19 cells nor oBRB tissue of porcine (Pelkonen et al. 2017; Zhang et al. 2017a). Mrp1 and Mrp4 were also detected in the basal membrane of the mouse RPE; in accordance, the efflux of [^{3}H]-zidovudine from the retina might be inhibited by MRP inhibitor MK571 (Chapy et al. 2016). Moreover, Mrp4 was reported to be also uniformly expressed in the physiologically developing retinal vasculature of the neonatal mouse including the capillaries and large vessels (Tagami et al. 2009). RCECs of the adult mouse also showed expression of Mrp3, Mrp4, and Mrp6 mRNA (Tachikawa et al. 2008).

Generally, MRPs efflux a wide variety of endogenous compounds and therapeutic drugs. For example, MRP4 mediates cellular efflux of both cAMP and prostaglandin E_2 (PGE_2), indicating involvement of MRP4 in angiogenesis. In human RCECs, VEGF was reported to dose-dependently decrease expression of MRP4 mRNA and protein. *Abcc4* knockdown using RNAi enhanced cell migration and attenuated serum starvation-induced cell apoptosis, assembled and aggregated into a massive tube-like structure (Tagami et al. 2010). Similarly, *Abcc4* deficiency did not cause overt abnormalities in the development of the retinal vasculature of mice, but retinal vascular development was suppressed in response to forskolin administration. The forskolin-treated *Abcc4*$^{-/-}$ mice showed an increased number of Ki67-positive and cleaved caspase 3-positive RCECs and significant decreases in the amount of pericyte coverage and number of empty sleeves. Moreover, following exposure of hyperoxia, the *Abcc4*$^{-/-}$ mice showed a significant increase in the unvascularized retinal area. These results indicate that MRP4 may have its protective roles in the retinal vascular development by regulating the intracellular cAMP level (Matsumiya et al. 2012).

Many kinds of drug transporters, such as OATPs, OATs, P-GP, BCRP, and MRPs, are expressed at the BRB, mediating transport of therapeutic drugs across the BRB. These transporters often overlap substrate specificity. Thus net effect is attributed to their interplay. For example, the elimination of p-aminohippuric acid, benzylpenicillin, and 6-Mercaptopurine from vitreous humor is mediated by several transporters. P-aminohippuric acid, benzylpenicillin, and 6-Mercaptopurine are

substrates of MRP4 (Uchida et al. 2007) and OAT3 (Hosoya et al. 2009), indicating that OAT3 and MRP4 in common contribute to the efflux transport of PAH, PCG, and 6-MP from the retina across the BRB. Another example is verapamil. Although P-GP is also highly expressed at the iBRB, P-GP has little involvement in the retinal uptake of verapamil, which is partly attributed to the existence of influx transporters (Chapy et al. 2015; Fujii et al. 2014). Transport of E17βG and dehydroepiandrosterone sulfate across BRB may be attributed to the combined effects of OATP1A4, OAT3, and MRP4 (Hosoya et al. 2011).

10.3 Alterations in BRB Function Under Disease Status and Clinic Significances

10.3.1 The BRB and Diabetic Retinopathy

Diabetic retinopathy, a complication of diabetes, is the leading cause of acquired blindness, which is involved in functional and structural changes of the BRB. Microvascular disorders are often diabetic retinopathy although other cells such as RPE cells are affected by diabetes. The vascular changes are clearly linked to the loss of visual acuity and clinical alterations in the retinal vasculature. Early vascular changes include leukostasis, aggregation of platelets, alteration in blood flow, degeneration of pericytes, and basement membrane thickening. The increased retinal vascular permeability associated with diabetic retinopathy may result from alterations in the tight junction and adherens junction complexes or from endothelial cell death. Macular edema is closely associated with the loss of visual acuity in diabetic retinopathy, which is attributed to the increased BRB permeability (Frey and Antonetti 2011; Arden and Sivaprasad 2011).

10.3.1.1 Inflammation and Diabetic Retinopathy

Retinal inflammation plays a major role in the pathogenesis of diabetic retinopathy. Hyperglycemia is considered as a pro-inflammatory environment via releasing some inflammatory cytokines [such as tumor necrosis factor-α (TNF-α), interleukin -1β (IL-1β), and interleukin-6 (IL-6)] and chemokine ligands (such as CCL2, CCL5, and CCL12)].These cytokines and chemokines induce the disorganization and redistribution of junctional proteins in microvasculature, leukocyte activation, release of intercellular adhesion molecule-1 (ICAM-1), and other cell adhesion molecules, in turn, increasing vascular permeability and further exacerbating the inflammatory milieu of the retina. For example, TNF-α downregulated expression of tight junction proteins (such as claudin-5 and ZO-1). Hyperglycemia upregulated levels of ICAM-1 and high mobility group box-1 (HMGB-1), which was in line with increases in leaky of Evans blue in rat retina (Ran et al. 2016). Streptozotocin (STZ)-induced

diabetes significantly upregulated expression of retinal CCL2 and increased monocyte trafficking in rats. In accordance with this, intraocular injection of the CCL2 into nondiabetic rats also increased retinal monocyte trafficking, indicating the ability of the chemokine to attract monocytes/macrophages into retinal tissue. High glucose exposure was also reported to upregulate CCL2 expression in human RCECs. Contrarily, CCL2 deficiency prevented the increase in vascular permeability and monocyte trafficking in the retinas of diabetic rats (Rangasamy et al. 2014). Moreover, diabetes also significantly increased levels of retinal cathepsin D and CCL2, accompanied by increases in vascular permeability to albumin in the retinas of mice. Patients with diabetic macular edema also showed increases in levels of serum cathepsin D protein. In human RCECs, cathepsin D was reported to disrupt endothelial junctional barrier via increasing RhoA/ROCK cell contractility (Monickaraj et al. 2016). Significant increases in expression of CXC chemokine platelet factor-4 (PF-4/ CXCL4) were also found in both vitreous fluid from patients with proliferative diabetic retinopathy and the retinas of diabetic rats. In human RCECs, it was found that PF-4/CXCL4, an angiostatic chemokine, inhibited VEGF-induced signal transduction and inhibited cell migration (Nawaz et al. 2013). Platelet factor-4 variant (PF-4var/CXCL4L1) was also reported to inhibit VEGF-mediated hyperpermeability. In accordance, intravitreal PF-4var/CXCL4L1 or bevacizumab attenuated diabetes-induced BRB breakdown in rats, leading to decreases in vascular leakage by approximately 70% and 73%, respectively, compared with phosphate buffer saline injection. These effects were also associated with upregulation of occludin and vascular endothelial-cadherin and downregulation of hypoxia-inducible factor (HIF)-1α, VEGF, TNF-α, receptor for advanced glycation end products (RAGE), and caspase-3 (Abu El-Asrar et al. 2016). Nuclear factor-κB (NF-κB) pathway is also involved in the pathogenesis of diabetic retinopathy. It was reported that administration of NF-κB inhibitor dehydroxymethylepoxyquinomicin suppressed retinal adherent leukocytes, expression of inflammatory molecules (ICAM-1 and VEGF), and renin angiotensin system (RAS)-related molecules such as angiotensinogen and angiotensin-II receptor 1 (AT1-R) induced by diabetes (Nagai et al. 2007).

10.3.1.2 Vascular Endothelial Growth Factor (VEGF) and Diabetic Retinopathy

VEGF, a pro-angiogenic growth factor secreted preferentially from the basal surface of the RPE and Müller cells, modulates and maintains the extracellular space in and around the Bruch's membrane and modulates the growth/density of endothelial cells in the choriocapillaris (Kay et al. 2013; Le 2017). Diabetic retinopathy is often associated with the increases in retinal VEGF levels (Kay et al. 2013; Lin et al. 2011; Nawaz et al. 2013). Animal and clinical trials have demonstrated that diabetes-induced increases in retinal VEGF levels are coincided with BRB breakdown (Le 2017).VEGF is involved in the pathogenesis of diabetic retinopathy via inducing retinal ICAM-1 expression, vascular permeability, leukostasis, BRB breakdown, or vascular lesions (Le 2017). In normal rats, it was found that $VEGF_{164}$/VEGF-A was

at least twice as potent as VEGF120 at inducing ICAM-1-mediated retinal leukostasis and BRB breakdown following intravitreous injections (Ishida et al. 2003). Moreover, blockade of endogenous VEGF-A with EYE001 significantly suppressed diabetes-induced retinal leukostasis and BRB breakdown, indicating that VEGF-A is an important isoform in the pathogenesis of diabetic retinopathy. Roles of VGEF in pathogenesis of diabetic retinopathy were confirmed by conditional VEGF knockout mice. It was found that conditional VEGF knockout significantly reduced leukostasis, expression of inflammatory biomarkers, depletion of tight junction proteins, numbers of acellular capillaries, and vascular leakage in the retina of diabetic mice (Wang et al. 2010). In general, VEGF shows its pathophysiologic effects in diabetic retinopathy in two ways (Deissler et al. 2014). One, VEGF increases vascular permeability and causes fluid extravasation and retinal edema via affecting endothelial tight junctions. Second, VEGF causes leukocyte aggregation in the retinal microvasculature, resulting in local cytokine production and inflammatory cell migration through the endothelium, both of which contribute to BRB breakdown. Anti-VEGF therapy with aflibercept, bevacizumab, or ranibizumab has been a hallmark strategy to prevent diabetic retinopathy in the clinic (Virgili et al. 2017), demonstrating efficiency of anti-VEGF drugs on the improvement of vision in people with diabetic macular edema. Some drugs such as brimonidine, memantine (Kusari et al. 2007, 2010), and corticosteroids (Edelman et al. 2005; Wang et al. 2008) also attenuate diabetic macular edema and retinopathy partly via affecting expression of retinal VEGF expression or downstream signal proteins of the VEGF receptor.

10.3.1.3 Hyperosmolar Stress and Diabetic Retinopathy

Diabetic retinopathy is associated with osmotic stress resulting from hyperglycemia and intracellular sorbitol accumulation. In vitro, it was reported that high glucose increased expression of the water channel aquaporin-1 (AQP1) and cyclooxygenase (COX)-2, increased activity of the osmolarity-sensitive transcription factor tonicity enhancer-binding protein (TonEBP), and enhanced endothelial migration and tubulization. These alterations by high glucose were reversed by AQP1 and TonEBP siRNA, indicating that high glucose-induced hyperosmolarity promotes angiogenesis and retinopathy via activating TonEBP (Madonna et al. 2016). It is generally accepted that glucose is reduced to sorbitol by aldose reductase, and sorbitol is eventually metabolized to fructose by sorbitol dehydrogenase. Intracellular sorbitol accumulation induces osmotic damage of the retinal vascular cells and RPE cells, loss of pericytes, basement membrane thickness, and oxidative stress, all of which contribute to iBRB rupture during diabetic retinopathy (Lorenzi 2007). RPE cells subjected to hyperosmolar stress also underwent osmoadaptative responses such as shrinkage of RPE cells, alterations in AQP expression, increases in VEGF expression, placental growth factor, monocyte chemoattractant protein-1, and basic fibroblast growth factor (Willermain et al. 2018), further impairing RPE function. Hyperosmolar conditions also increases activity and expression of TAUT in RPE

cells, leading to increases in the uptake of taurine (El-Sherbeny et al. 2004). Moreover, hyperosmolar condition also increased expression of aldose reductase (Winges et al. 2016), in turn, further enhancing sorbitol accumulation.

10.3.1.4 Plasma Kallikrein-Kinin System (PKKs) and Diabetic Retinopathy

Accumulating evidences have demonstrated contribution of plasma kallikrein-kinin system (PKKs) to diabetic retinopathy. It was reported that levels of plasma prekallikrein and plasma kallikrein in vitreous from subjects with diabetic macular edema were increased to 2.0-fold and 11.0-fold, respectively, of those with a macular hole (Kita et al. 2015). In normal rats, intraocular injection of bradykinin dose-dependently might increase plasma extravasation, which was inhibited by bradykinin receptor 2 antagonist Hoe140 (Abdouh et al. 2008; Phipps et al. 2009). Similarly, the bradykinin receptor 2 agonist bradykinin might vasodilate retinal vessels in a concentration-dependent manner, which was completely blocked by Hoe140. But bradykinin receptor 1 agonist des-Arg9-bradykinin had no this effect. However, in diabetic rats, des-Arg9-bradykinin could also produce a concentration-dependent vasodilatation and was also inhibited by the bradykinin receptor 1 receptor antagonist des-Arg10-Hoe140 (Abdouh et al. 2003), which may be explained by these findings that the bradykinin receptor 1 in the retina is only minimally expressed under physiological conditions, but diabetes significantly upregulated expression of bradykinin receptor 1 without affecting bradykinin receptor 2 (Abdouh et al. 2008; Kita et al. 2015; Pouliot et al. 2012). Animal experiments have been demonstrated that diabetes-induced retinal vascular permeability is attenuated by plasma kallikrein inhibitor or bradykinin receptor antagonists (Abdouh et al. 2008; Catanzaro et al. 2012; Clermont et al. 2011; Pouliot et al. 2012) or plasma prekallikrein-gene deficiency (Kita et al. 2015). FOV-2304, a non-peptide selective bradykinin receptor 1 antagonist, also blocked retinal vascular permeability, inhibited leukocyte adhesion, and abolished the retinal mRNA expression of several inflammatory mediators in diabetic rats (Pruneau et al. 2010). These results indicate that the blockade of the PKKs is a promising therapeutic strategy for diabetic retinopathy.

Interestingly, the response of plasma kallikrein following injection into vitreous of diabetic rats was blocked by bradykinin receptor antagonist but not by bevacizumab. In mice, administration of VEGF receptor 2 antibody DC101 did not affect bradykinin-induced retinal thickening. Moreover, although increased VEGF levels were also observed in diabetic macular edema vitreous, no correlation of plasma kallikrein level and VEGF level was found. These results indicate that diabetic macular edema induced by PKKs is VEGF-independent (Kita et al. 2015). However, a report showed that plasma prekallikrein-gene deficiency partly decreased the VEGF-induced retinal vascular permeability and retinal thickening or TNFα-induced retinal thickening in mice. Systemic administration of plasma kallikrein inhibitor VA999272 also reduced VEGF-induced retinal thickening in both mice and rats, indicating that plasma kallikrein is required for the full effects of VEGF on retinal

vascular permeability and retinal thickening (Clermont et al. 2016). Retinal hemorrhages also occur under diabetic condition. Intravitreal injection of autologous blood was reported to induce retinal vascular permeability and retinal leukostasis, which were ameliorated by plasma kallikrein inhibition. Intravitreal injections of exogenous plasma kallikrein also induced retinal vascular permeability, leukostasis, and retinal hemorrhage, indicating that retinal hemorrhage increases retinal vascular permeability and leukostasis partly via plasma kallikrein (Liu et al. 2013).

Real mechanisms that PKKs induce retinal vascular permeability and retinal thickening are not fully understood. Bradykinin was reported to evoke intracellular Ca^{2+} transients in primary human RPE, which was enhanced by pretreatment with TNF-α and/or IL-1β but inhibited by fasitibant chloride, a selective bradykinin receptor 2 antagonist. TNF-α and/or IL-1β enhanced bradykinin-induced Ca^{2+} response via increasing expression of both bradykinin receptor 2 and COX-2, as well as secretion of prostaglandin E_1 and E_2 into the extracellular medium (Catalioto et al. 2015). Intravitreal injections of either plasma kallikrein or collagenase, but not bradykinin, also induced retinal hemorrhage in rats. Proteomic analysis showed that plasma kallikrein increased collagen degradation in pericyte-conditioned medium and purified type IV collagen, indicating that plasma kallikrein leads to breakdown of BRB due to its collagenase-like activity (Liu et al. 2013). However, intravitreal injection of bradykinin also increased retinal vascular permeability, which might be prevented by both vasoinhibins and Hoe-140. In ARPE-19, bradykinin also increased permeability of the BRB and decreased endothelial monolayer resistance, which was attributed to redistribution of actin cytoskeleton, subsequently reorganization of tight and adherens junctions. These effects were reversed by NO synthase inhibitor L-NAME, vasoinhibins, and N-acetyl cysteine (Arredondo Zamarripa et al. 2014). Bradykinin could also inhibit TGF-β1-stimulated ARPE-19 cell proliferation, collagen I, fibronectin, and MMP-2 secretion as well as Akt phosphorylation via activating bradykinin receptor 2 (Cai et al. 2016). In normal rats, bradykinin-induced vasodilatation was involved in intracellular $Ca2^{+}$ mobilization and products of the cyclooxygenase-2 (COX-2) pathway, but in STZ-diabetic rats, the vasodilatation in response to des-Arg9-bradykinin was involved in both calcium influx and intracellular calcium mobilization, which was blocked by $GdCl_3$, 2,5-di-t-butylhydroquinone, and cADP ribose (Abdouh et al. 2003).

10.3.1.5 Renin Angiotensin System (RAS) and Diabetic Retinopathy

Clinical and experimental studies have demonstrated that abnormalities of the renin angiotensin system (RAS) may play a significant role in the progression of the diabetic retinopathy, presumably through local changes in the blood flow and the production of angiotensin II (Ang II) (Ola et al. 2017; Phipps et al. 2012). Besides circulating RAS, RAS locally exists in the retina. Animal experiments have demonstrated that diabetes increases components of RAS including prorenin, angiotensinogen, Ang II, angiotensin-converting enzyme (ACE), angiotensin-converting enzyme 2 (ACE2), and Ang II receptor 1 (AT1R) in the retina (Ola et al. 2017; Nagai et al. 2007; Verma et al. 2012). Several reports have shown that

vitreous pool of proliferative diabetic retinopathy patients also exhibited an increased level of Ang II and other RAS components compared to nondiabetic subjects (Ola et al. 2017; Phipps et al. 2012). Rodent experiments have aslo revealed that ACE inhibitors and AT1R blockers could reduce diabetes-induced retinal microvascular damage with reductions in vascular leakage, decreased formation of acellular capillaries, and decreased expression of angiogenic factors such as VEGF (Mori et al. 2002; Wilkinson-Berka et al. 2007; Zhang et al. 2007). ACE inhibition or AT1R blockade also decreased diabetes-induced retinal leukostasis and upregulation of adhesion molecules (Mori et al. 2002; Chen et al. 2006; Silva et al. 2007), but AT2R blockade did not show this effect (Nagai et al. 2007).

AT1R antagonist candesartan could decrease diabetes-induced or Ang II-stimulated retinal vascular permeability without affecting retinal vascular permeability in normal rats although candesartan decreased hypertension both in diabetic and normal rats (Phipps et al. 2009). Ang II infusion also significantly increased expression of plasma kallikrein in rat retina. Bradykinin receptor 2 antagonist Hoe140 and plasma kallikrein inhibitor (ASP-440) might attenuate Ang II-induced retinal vascular permeability, indicating that activation of AT1R increases retinal vascular permeability partly via PKKs (Phipps et al. 2009). Clinical trial (Mauer et al. 2009) demonstrated effects of ACE inhibitor enalapril and the AT1R inhibitor losartan on progression of diabetic retinopathy to a similar extent, whose odds of retinopathy progression were reduced by 65% with enalapril treatment and 70% with treatment, respectively. Data from 5321 diabetic patients demonstrated that treatment with candesartan significantly reduced the incidence of development of diabetic retinopathy in type 1 diabetics without affecting its progression (Chaturvedi et al. 2008). But in type 2 diabetes, treatment with candesartan reduced the development of retinopathy and even resulted in a 34% regression of retinopathy compared with the control group (Sjølie et al. 2008). Taken together, these results suggest that RAS blockade may be useful for slowing progression and decreasing the severity of diabetic retinopathy.

ACE2 is expressed in the retina, which converts the vasoconstrictive and pro-inflammatory peptide Ang II into the vasodilatory and anti-inflammatory peptide angiotensin-(1-7) [Ang(1-7)], which exhibits its effects primarily through the receptor Mas. A report showed that STZ-induced diabetes significantly increased mRNA levels of the vasodeleterious axis of the RAS (angiotensinogen, renin, pro/renin receptor, ACE, and AT1R) in the retina of *eNOS*$^{-/-}$ mice, leading to increases in ratios of ACE/ACE2 and AT1R/Mas mRNA levels by approximately tenfolds and threefolds, respectively (Verma et al. 2012). The increases in vascular permeability, infiltrating CD45-positive macrophages, and activation of CD11b-positive microglial cells as well as formation of acellular capillaries in the retina of diabetic mice were attenuated by intraocular administration of adeno-associated virus-ACE2 or Ang-(1-7) vector (Verma et al. 2012). Intraocular administration of adeno-associated virus-ACE2 vector was also reported to attenuate the increase of acellular capillaries and leaky of macrophages/microglia in the retina of STZ-induced diabetic mice (Dominguez et al. 2016). Similarly, in STZ-induced diabetic rat retinas, the

increased numbers of acellular capillaries were almost completely prevented by gene delivery of either ACE2 or Ang-(1-7) (Verma et al. 2012).

Diabetes is often associated with hypertension, and patients with hypertension are at a greater risk of developing diabetic complications including retinopathy, inferring that therapeutic effects of RAS blockade on diabetic retinopathy are also attributed to the reduction in blood pressure. Clinical trials have demonstrated no different effects on progression of retinopathy between captopril and atenolol treatments or between enalapril and nisoldipine in type 2 diabetes (Phipps et al. 2012). In consistence, diabetic spontaneously hypertensive rats (SHR) showed significantly higher number of ED1/microglial-positive cells and the expression of ICAM-1 in the retina than in control SHR. The SHR also possessed higher NF-κB p65 levels than Wistar Kyoto (WKY) rats. These abnormalities in diabetic SHR rats were completely prevented by losartan or complex of hydralazine+ reserpine +hydrochlorothiazide) (Silva et al. 2007). Similarly, SHR rats showed higher number of BrdU-positive retinal cells than WKY rats. A significant reduction in cell replication was found only in diabetic SHR, and this reduction was associated with enhanced $p27^{Kip1}$, fibronectin, and VEGF retinal expressions and greater blood-retinal barrier breakdown (Lopes et al. 2008). These results indicate contribution of concomitant diabetes and hypertension to diabetic retinopathy. However, a rat experiment showed that ramipril, losartan, and nifedipine showed similar antihypertensive efficiencies, but ramipril and losartan showed stronger decreases in retinal leukostasis and expression of ICAM-1 induced by diabetes than nifedipine, indicating that their effects seem to be partly independent of blood pressure and to be associated with a decrease in ICAM-1 gene expression (Chen et al. 2006), which need further investigation.

It is worth noting that systemic Ang II reduces RPE renin production via stimulating AT1R and that systematic application of ACE inhibitors strongly activates local RAS in the retina, indicating that the systemic treatment with RAS blockade for retinal degeneration and systemic disease may cause side effects detrimental, or perhaps beneficial, to retinal disease (Strauß 2016) via affecting retinal RAS.

10.3.1.6 Alterations in Transport under Diabetic Status

10.3.1.6.1 Alterations in Glucose Transport

The retina and RPE are highly metabolically active tissues with substantial demands for glucose. In STZ-induced diabetic rats, Glut1 expression in retinal microvessels but not in RPE was decreased by approximately 50%, without altering the retina microvascular density, indicating that the fraction of the glucose entering the retina of diabetes is likely to be greater across the RPE than across the retinal vasculature (Badr et al. 2000). Decreases in expressions of Glut1 proteins not mRNA were also found in the retinas of diabetic GK rats and alloxan-treated rabbits (Fernandes et al. 2004). In TR-iBRB cells, high glucose exposure also decreased expression of Glut1 protein. Moreover, higher content of high molecular weight ubiquitin conjugates

was found in both membrane fractions of diabetic retinas and endothelial cells treated with high glucose exposure, indicating that the decreased expression of Glut1 protein may be associated with its increased degradation by a ubiquitin-dependent mechanism (Fernandes et al. 2004). Importantly, although expressions of Glut1 on the luminal plasma membrane of the RCECs and in homogenates of the whole retina in diabetic rats were significantly decreased (about 55% and 36% of control rats, respectively) (Tang et al. 2000), retinal glucose levels were significantly elevated by fourfold to sixfold compared with the nondiabetic rats. It was also found that glucose influx increased with increasing plasma glucose in both diabetic and normal rats (Puchowicz et al. 2004). Dehydroascorbic acid transport across BRB is mediated by GLUT1; it was consistent with the decreases in Glut1 expression that [^{14}C] dehydroascorbic acid transport across the BRB in STZ-induced diabetic rats was less than 35% of normal rats, inferring that hyperglycemia reduces the supply of vitamin C to the retina (Minamizono et al. 2006).

10.3.1.6.2 Alterations in H_2O Transport

BRB maintains fluid homeostasis in the retina by removing fluid out of the retina via the retinal vasculature. Besides the ionic and osmotic gradients, transport H_2O across BRB is mainly mediated by aquaporins (AQPs). Thirteen AQPs have been identified in mammals, most of which are expressed in the retina. Diabetes significantly altered the expression and distribution of retinal AQPs in the retina (Xia and Rizzolo 2017) in a species-dependent manner. For example, diabetes upregulated AQP5, 9, 11, and 12 but downregulated AQP0 in the RPE of rats (Hollborn et al. 2011). Diabetic retinopathy is often associated with cellular stressors (such as hypoxia and oxidative stress). In primary cultures of human RPE, it was found that exposure of stressors (chemical hypoxia, oxidative stress, VEGF, and high glucose) upregulated expression of AQP9 (Hollborn et al. 2012). AQP9, an aquaglyceroporin, is not only permeable to water but also to non-charged solutes, such as lactate. AQP9 expression is considered to be required for L-lactate to maintain retinal neuronal survival (Akashi et al. 2015), indicating that the upregulation of AQP9 in RPE cells may prevent lactic acidosis and subretinal edema under ischemic and oxidative stress conditions (Akashi et al. 2015; Hollborn et al. 2012). Hyperosmolarity could alter AQP expression. It was reported that 10 min exposure to an osmolar stress (400 mM sucrose or 200 mM NaCl) significantly decreased AQP4 expression in ARPE-19 cells (Willermain et al. 2014b). Under normal condition, expression of AQP1 in the rat retina is at minimal levels, diabetes increased expression of AQP1, and hypertension also enhanced expression of AQP4 under diabetic conditions, which were reversed by valsartan and metoprolol (Qin et al. 2012). Furthermore, intravitreal injection of VEGF also increased AQP4 expression in the retina of normal and diabetic rats. TGN-020, a selective AQP4 inhibitor, suppressed VEGF-induced enlargement of Müller cells and increases in intracellular levels of NO. Thus, the authors gave a conclusion that VEGF induced Müller cell swelling through the formation of NO and AQP4 channels (Kida et al. 2017). Importantly, although

diabetes or administration of VEGF increased AQP4 expression in the retina of rats (Cui et al. 2012; Kida et al. 2017), AQP4 downregulation exacerbated diabetic retinopathy and aggravated inflammatory response (Cui et al. 2012). Thus, the cellular mechanisms mediating expression of AQPs by diabetes and their clinic significances need further investigation.

10.3.1.6.3 Alterations in Active Transport

Tight junctions maintain the ion gradients essential for transcellular transport mechanisms to function. Diabetes can directly decrease the ion gradients via decreasing $Na^{+/}K^{+}$-ATPase activity, contributing to retinal edema (Xia and Rizzolo 2017). Transport of L-lactic acid across the RPE is dependent on a pH gradient. Intracellular pH is regulated by $Na^{+}/HCO3^{-}$ cotransporters. The Na^{+}-dependent movement of HCO_3^{-} is actively driven by the physiologic Na^{+} gradient established by the Na^{+}/K^{+}-ATPase. Thus, inadequate activity of the Na^{+}/K^{+}-ATPase impaired transport of L-lactic acid. Taurine is the most abundant free amino acid in the retina, which functions as an antioxidant and may attenuate the spread of cell death in RPE cells. Diabetes and high glucose downregulate expression and activity of retinal TAUT (Zeng et al. 2010; Lee and Kang 2013) partly due to overexpression of aldose reductase (Nakashima et al. 2005b; Stevens et al. 1999). Taurine deficiency can lead to severe damage to photoreceptors (Ripps and Shen 2012). Thus, decreases in transport of retinal taurine by diabetes and subsequent taurine depletion from the retina could contribute to visual impairment.

10.3.1.6.4 Alterations in ABC Transporters

Some diseases may affect expression of retinal P-GP and BCRP. A report showed that 24-week STZ-induced diabetic mice demonstrated lower expression of retinal P-GP and BCRP, with the breakdown of the iBRB, which might be linked to the pathogenesis of early diabetic retinopathy (Li et al. 2017). In D407 cells, it was reported that high glucose exposure significantly decreased P-GP expression of both mRNA and protein levels, attenuated P-GP activity, and increased expressions of both mRNA and protein of inducible nitrate oxide synthase (iNOS). High glucose exposure also decreased expression of pregnane X receptor (PXR) mRNA. These alterations by high glucose were partially blocked by a selective iNOS inhibitor, whose effects were antagonized with the addition of L-arginine, a substrate for NO synthesis. These results demonstrate roles of iNOS induction in decreased P-GP expression and function at the human oBRB under hyperglycemic conditions (Zhang et al. 2012).

Diabetes is often associated with oxidative stress, which is a contributing factor to RPE cell dysfunction in diabetic retinopathy and age-related macular degeneration. In general, cellular antioxidants in RPE also play a critical role in combating oxidative stress; among the cellular antioxidant constituents, reduced glutathione

(GSH) plays a significant role in cellular defense against pro-oxidants. MRP1, expressed in PRE, regulates levels of cellular GSH via efflux GSH. In ARPE-19 cells, it was found that MRP inhibitors (MK571 and sulfinpyrazone) significantly decreased GSH efflux by 50%. MRP1 silencing using MRP1-specific siRNA also caused a significant 60% reduction in GSH efflux. Moreover, the downregulation of MRP1 developed to resistance to H_2O_2-induced cell death. Contrarily, overexpression of MRP1 was susceptible to H_2O_2-induced cell death, which was consistent with lower cellular GSH levels in MRP1 overexpressed cells (Sreekumar et al. 2012). These results indicate that MRP1 shows its roles in oxidant-induced cell death via efflux GSH and that MRP1 may be a potential therapeutic target in pathological retinal degenerative disorders linked to oxidative stress.

10.3.2 Ischemia-Reperfusion/Hypoxia and BRB Breakdown

Retinal ischemia-reperfusion injury is associated with many ocular diseases such as acute retinal vein occlusion, diabetic retinopathy, and glaucoma. Oxidative injury is one of the complications after retinal ischemia-reperfusion injuries, accompanied by retinal swelling, neuronal cell death, and glial cell activation, due to oxidation stress and release of inflammatory cytokines and chemokines (Kaur et al. 2008). Ischemia-reperfusion was reported to significantly increase retinal mRNA expression of several pro-inflammatory cytokines such as IL-1β (3.2-fold), IL-6 (4.2-folds), TNF-α (5.6-fold), and CCL2 (116.9-folds) in the retinas, accompanied by significant increases in leaky of Evans Blue (3.8-fold) and number of CD45-positive cells (Gonçalves et al. 2016). The roles of TNF-α in cytokine-induced BRB breakdown were demonstrated in TNF-α-deficient mice. It was found that compared with wild-type mice, TNF-α deficiency significantly reduced leukocyte accumulation in retinal vessels by 80%, 100%, and 100% after intravitreous injection of VEGF, IL-1β, and platelet-activating factor (PAF), respectively. The absences of TNF-α significantly reduced retinal vascular permeability induced by injection of PAF, but not VEGF nor IL-1β. Moreover, TNF-α deficiency significantly reduced leukostasis and mild reduction in vascular leakage, but did not affect hypoxia-induced retinal neovascularization (Vinores et al. 2007). Ischemia-reperfusion was also reported to induce occludin Ser490 phosphorylation and ubiquitination, distribution of specific tight junction proteins, and activation and phosphorylation of VEGF receptor-2 (VEGFR-2) at tyrosine 1175, all of which contribute to the increased vascular permeability. Intravitreal injection of bevacizumab prevented VEGFR-2 activation, occludin phosphorylation, and vascular permeability induced by ischemia-reperfusion (Muthusamy et al. 2014). Furthermore, ischemia-reperfusion also altered distribution of glial fibrillary acidic protein (GFAP) and AQP4 in the retina. In normal rats, expression of GFAP was confined to astrocytes in ganglion layer; ischemia-reperfusion markedly increased expression of GFPA, whose expression was not just confined to the ganglion layer but also found in the Muller cell processes. AQP4 staining is discontinuous along the blood vessels and appeared weak in normal rats. AQP4

immunoreactivity surrounding retinal vessels became more intense in the ischemia-reperfusion retina (Li et al. 2011). The increased expression of AQP4 and alterations in its distribution led to increases in transport of water from blood vessels to the retinal tissues, contributing to edema formation in hypoxic conditions (Kaur et al. 2008; Li et al. 2011). Moreover, under normal conditions, Müller cells do not express GFAP, but many insults including hypoxia to the retina lead to a rapid upregulation of GFAP in these cells. Upregulation of GFAP in stressed Müller cells is often associated with an upregulation of heat shock proteins and alterations in cytoskeletal protein synthesis, swelling of Müller/astrocytes cells, and vascular leakage in hypoxic condition (Kaur et al. 2008). Caveolae-mediated vesicular transport is a system of transcellular transport of macromolecules in the BRB. Caveolin-1 is a principal structural component of caveolae. Under normal condition, expression of caveolin-1 in the RCECs contributes to the integrity of the BRB. Hypoxia increased expressions of caveolin-1 mRNA and protein and caveolin-1 siRNA significantly reduced hypoxia-induced albumin leakage in the retinas and neovascularization, which was in line with reduction of caveolin-1 mRNA and protein, indicating that overexpression of caveolin-1 in the retina is associated with BRB breakdown and neovascularization formation (Tian et al. 2012).

BRB breakdown by hypoxia is linked to induction of hypoxia-inducible factor (HIF)-1 in Müller cells. In vitro, it was found that unlikely to wild-type mice, hypoxia did not induce expression of abundance of VEGF in Müller cells from conditional Hif-1α knockout mice, which was consistent with decreases in expression of HIF-1, indicating that HIF-1α in Müller cells is a major mediator of ischemia-/hypoxia-induced VEGF overproduction in the retina. In vivo, hypoxia induces expression of HIF-1, VEGF, ICAM-1, vascular leakage, and retinal neovascularization in mice, which may be attenuated by conditional HIF-1α deficiency. Moreover, HIF-1α deficiency also reverses the diabetes-induced inflammation, vascular leakage, and leucostasis (Lin et al. 2011), indicating that Müller cell-derived HIF-1α is therefore a promising therapeutic target for diabetic retinopathy.

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Chapter 11
Contributions of Drug Transporters to Blood-Placental Barrier

Li Liu and Xiaodong Liu

Abstract The placenta is the only organ linking two different individuals, mother and fetus, termed as blood-placental barrier. The functions of the blood-placental barrier are to regulate material transfer between the maternal and fetal circulation. The main functional units are the chorionic villi within which fetal blood is separated by only three or four cell layers (placental membrane) from maternal blood in the surrounding intervillous space. A series of drug transporters such as P-glycoprotein (P-GP), breast cancer resistance protein (BCRP), multidrug resistance-associated proteins (MRP1, MRP2, MRP3, MRP4, and MRP5), organic anion-transporting polypeptides (OATP4A1, OATP1A2, OATP1B3, and OATP3A1), organic anion transporter 4 (OAT4), organic cation transporter 3 (OCT3), organic cation/carnitine transporters (OCTN1 and OCTN2), multidrug and toxin extrusion 1 (MATE1), and equilibrative nucleoside transporters (ENT1 and ENT2) have been demonstrated on the apical membrane of syncytiotrophoblast, some of which also expressed on the basolateral membrane of syncytiotrophoblast or fetal capillary endothelium. These transporters are involved in transport of most drugs in the placenta, in turn, affecting drug distribution in fetus. Moreover, expressions of these transporters in the placenta often vary along with the gestational ages and are also affected by pathophysiological factor. This chapter will mainly illustrate function and expression of these transporters in placentas, their contribution to drug distribution in fetus, and their clinical significance.

Keywords Blood-placental barrier · Syncytiotrophoblast · Transplacental transport · SLC transporter · ABC transporters · Fetus drug distribution

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11.1 General Introduction

The placenta is the only organ linking two different individuals, mother and fetus. Its main functional units are the chorionic villi within which fetal blood is separated by only three or four cell layers (placental membrane) from maternal blood in the surrounding intervillous space (Fig.11.1), termed as "blood-placental barrier." The functions of the blood-placental barrier are to regulate material transfer between the maternal and fetal circulation, providing oxygen, water, carbohydrates, amino acids, lipids, vitamins, and other nutrients to the fetus while removing carbon dioxide and other waste products. The shape and structure of the placenta vary greatly from one species to another. In human, the maternal and fetal blood are separated by a single syncytiotrophoblast cell layer; thus human placentas are classified to be hemochorial placenta (Fig.11.1). In contrast, in mouse and rat, maternal and fetal blood are separated by three cell layers (cytotrophoblast, syncytiotrophoblast I, and syncytiotrophoblast II) (Han et al. 2018), whose placentas are classified to be hemotrichorial placentas (Fig.11.1). Importantly, structure and function of the placenta also vary during the gestational period. In early pregnancy, the main function of the placenta is to mediate implantation of the embryo into the uterus and produce hormones that prevent the end of the ovarian cycles. Immediately after successful implantation, the trophoblast cell layer of the blastocyst proliferates more rapidly

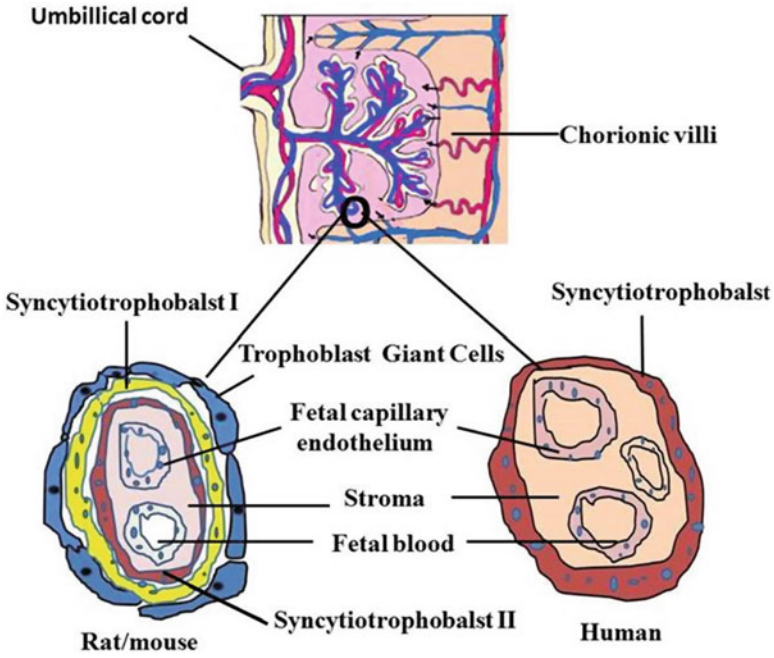

Fig. 11.1 Structures of human and rat/mouse placentas and function unit of fetus-derived chorionic villi

than the embryo and differentiates via two main pathways villous and extravillous pathways (Gude et al. 2004; Tomi et al. 2011). Extravillous cytotrophoblasts invade the deciduas to attach the placenta to the uterus and to remodel the maternal spiral arteries. Villous cytotrophoblasts differentiate and fuse to form syncytiotrophoblasts. In first trimester of pregnancy, maternal blood flow to the placenta is often limited because maternal spiral arteries are plugged by endovascular cytotrophoblasts. Nourishment is only obtained from the eroded maternal tissues; thus the placenta grows under a low oxygen environment. During the period, the fetus is generally most susceptible to damage from teratogens. By the end of first trimester, maternal blood begins to flow from the maternal spiral arteries into the intervillous space, and then the blood-placental barrier formed by a layer of syncytiotrophoblasts actually works as an interface of maternal-fetal exchange. With advancing gestation, the placental barrier or maternal-fetal diffusion distance gradually thins. The distance decreases from over 50 μm at the late second month to less than 5 μm by the 37th week of pregnancy, when the placental barrier only consists of an endothelial cell layer, thin layer of connective tissue, and a continuous syncytiotrophoblast with some individual trophoblasts underneath (Vähäkangas and Myllynen 2009). The number of microvilli increases around the beginning of the third trimester, the surface area available for maternal-fetal exchange reaches 13 m^2, villous vessels contain 25% of total fetoplacental blood volume (Kingdom et al. 2000; Luckhardt et al. 1996), and maternal blood supply to the placenta is about 30% of the mother's cardiac output, which itself increases by 30–40% (Khong et al. 1986; Gude et al. 2004; Tomi et al. 2011).

Transplacental transport includes passive diffusion, pinocytosis, and facilitated and active transport via transporter proteins (transporters). Both syncytiotrophoblast and fetal capillary endothelium express transporters. In syncytiotrophoblast, transporters are expressed in the brush border (apical membrane) facing maternal blood or basolateral membrane close to fetal capillaries. The identified ATP-binding cassette (ABC) transporters in human placenta include P-glycoprotein (P-GP/ABCB1), multidrug resistance-associated proteins (MRP1/ABCC1, MRP2/ABCC2, MRP3/ABCC3, MRP4/ABCC4, MRP5/ABCC5), and breast cancer resistance protein (BCRP/ABCG2). The identified solute carrier (SLC) protein transporters related to drug transport in human placenta include organic anion-transporting polypeptides (OATP4A1/SCLO4A1, OATP1A2/SLCO1A2, OATP1B1/SCLO1B1, and OATP3A1/SLCO3A1), organic anion transporters (OAT4/SCL22A11), organic cation transporters (OCT3/SLC22A3, OCTN1/SLC22A4, and OCTN2/SLC22A5), multidrug and toxin extrusion 1 (SLC47A1/MATE1), and equilibrative nucleoside transporters (ENT1/SLC29A1 and ENT2/SLC29A2). Most of the ABC efflux transporters identified in the placenta are expressed on the apical membrane of syncytiotrophoblast, some of which also are expressed on the basolateral membrane of syncytiotrophoblast or fetal capillary endothelium (Fig. 11.2). Moreover, expressions of these transporters in the placenta vary during the gestational period. Characteristics of these transporters and their substrates/inhibitors have been discussed in Chaps. 2 and 3. This chapter will focus on function and expressions of these transporters in placentas and its clinical significance.

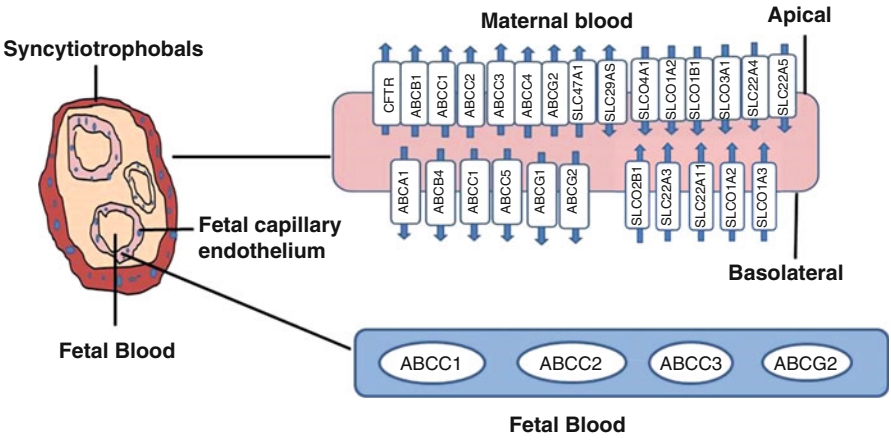

Fig. 11.2 Locations of main ABC and SLC transporters in human placenta

11.2 P-Glycoprotein (P-GP/ABCB1) and Blood-Placental Barrier

Extensively studied ABC transporter in the placenta is P-GP, coded by *ABCB1* gene. P-GP is mainly expressed in apical membrane of human and animal syncytiotrophoblast at all gestational ages (Ceckova-Novotna et al. 2006; Sun et al. 2006; Kalabis et al. 2005). Functions of P-GP are to efflux substrates from the syncytiotrophoblast back into the maternal circulation, preventing factors present in the maternal blood from entering the fetal circulation, which has been confirmed by series of in vivo and in vitro data. Both primary cultures of human trophoblast and BeWo cell lines (deriving from human placental choriocarcinoma) are widely employed to assess the function of placental P-GP using uptake studies with special P-GP substrates (such as digoxin and vinblastine) in presence of P-GP inhibitors (Utoguchi et al. 2000; Song et al. 2013). P-GP is highly expressed on the brush border membrane of BeWo cells and isolated trophoblast cells (Ushigome et al. 2000). In BeWo cells, the transports of digoxin, vinblastine, and vincristine in the basolateral-to-apical are greater than those in the apical-to-basolateral direction. Addition of cyclosporine A significantly increased the apical-to-basolateral penetration of these substrates (Ushigome et al. 2000). Dually perfused human placenta demonstrated that the fetal-to-maternal transfer of saquinavir was 108-fold higher than transfer from the maternal to fetal direction. Valspodar (PSC833) or elacridar (GF120918) significantly increased the placental transfer of saquinavir by 7.9-fold and by 6.2-fold, respectively. The P-GP expression was also correlated with the valspodar-induced change in the saquinavir transfer (Mölsä et al. 2005). Similarly, in human placenta slice, it was found that addition of verapamil significantly increased the apical-to-basolateral penetration of rhodamine 123 to 11-fold of that without verapamil (Song et al. 2013). These results demonstrate function of P-GP in the

BeWo cells and placenta. In vivo function of placentas has been demonstrated in *Abcb1* knockout mice (Smit et al. 1999; Lankas et al. 1998). It was reported that *Abcb1*$^{-/-}$ mice showed increased sensitivity to avermectin-induced teratogenicity (Lankas et al. 1998). When pregnant dams were exposed to the teratogenic isomer of avermectin, fetuses deficient in *Abcb1*$^{-/-}$ gene were 100% susceptible to cleft palate, whereas their (*Abcb1*$^{+/-}$) littermates were less sensitive (30% of fetuses with cleft palate). The homozygotes (*Abcb1*$^{+/+}$) fetuses were totally insensitive at the tested doses. Intravenous administration of the P-GP substrate drugs [^{3}H] digoxin, [^{14}C]saquinavir, or [^{14}C]paclitaxel to pregnant dams revealed that 2.4-, 7-, or 16-fold more drug, respectively, entered the *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ fetuses than entered wild-type fetuses (Smit et al. 1999). The placenta P-GP activity was completely inhibited by oral administration of the P-GP blockers valspodar or elacridar to heterozygous mice (Smit et al. 1999). In consistence, combination of ivermectin (a substrate of P-GP) and verapamil severely affected fetal genetic material and development and induced genotoxic effect in somatic cells of the dams in rats (el-Ashmawy et al. 2011). Placental P-GP was also proved to limit saquinavir penetration to fetus in clinical study (Huisman et al. 2001). All the studies demonstrate that P-GP plays an important role in limiting fetal penetration of various potentially harmful or therapeutic compounds, providing protection to the fetus.

Several evidences have demonstrated that P-GP expression is highest in early gestation, and then expression of placental P-GP decreases dramatically with gestational age (Gil et al. 2005; Kalabis et al. 2005; Mathias et al. 2005; Petrovic et al. 2015; Sun et al. 2006). Gil et al. (2005) reported a significant and progressive twofold decrease in expression of placental P-GP between early gestation (13–14 weeks) and late full term (38–41 weeks). Similarly, Sun et al. (2006) reported that levels of P-GP mRNA and protein in tissues of early gestation (7–13 weeks) were significantly higher than those found in term tissues (38–41 weeks). Moreover, levels of P-GP mRNA and protein in tissues of midgestation (24–35 weeks) were also higher than those in term but lower than those at early gestation (7–13 weeks). Mathias et al. (2005) reported that expression of P-GP, when normalized to alkaline phosphatase activity in 60–90-day placentas, was 44.8-fold higher than that in term placentas. When normalized to total protein, P-GP expressions in 60–90-day placentas and in 90–120-day placentas were 5.1-fold higher and 1.5-fold higher than that in term placentas, respectively, although no statistical difference between 90–120-day placentas and 60–90-day placentas or term placentas was obtained (Mathias et al. 2005). Williams et al. (2012) compared expressions of P-GP mRNA and protein in early first-trimester pregnancy (mean 8.8 weeks), early second-trimester pregnancy (mean 12.9 weeks), and normal term pregnancy (mean 39.4 weeks). They found that that P-GP expression detected using immunohistochemistry in early second-trimester pregnancy and in term placental samples were 78.5% and 52.5% of those in early first-trimester pregnancy, respectively. The measured levels of P-GP mRNA (mean ratio to reference gene) by QT-PCR in early first-trimester pregnancy, early second-trimester, and term placental samples were 63.74, 96.22, and 7.27 (Williams et al. 2012), respectively. Evseenko et al. (2006) also reported that that expression of P-GP mRNA and protein decreased

during trophoblast differentiation/syncytialization. Kalabis et al. (2005) compared expression of placental Abcb1b mRNA and P-GP from pregnant mice at embryonic day 9.5, 12.5, 15.5, 18.5, and 19. They found that both placental Abcb1a and Abcb1b mRNA were detected in trophoblast cells at all gestational ages and expression of placental Abcb1b mRNA was higher than those of placental Abcb1a mRNA. Expressions of placental Abcb1a and Abcb1b mRNA as well as P-GP peaked at embryonic day 12.5 and then progressively decreased toward term. Coles et al. (2009a) reported that P-GP levels in the placenta of CD-1 mice collected at midgestation (day 13) was 70% greater than in late-gestation (gestation day 18). In consistence, following intravenous administration, [^{3}H]saquinavir placenta-to-plasma and fetal-to-plasma ratios were significantly greater in late-gestation mice than in midgestation (Coles et al. 2009a).

Real mechanisms leading to the decreases in placental P-GP are not fully understood. In general, levels of steroid hormones vary along with gestation. Mouse experiment showed that maternal progesterone concentrations decreased with advancing gestation. The Abcb1b mRNA expression was reported to be positively correlated to maternal plasma progesterone concentration (Kalabis et al. 2005), demonstrating a potential role of progesterone in regulation of placental Abcb1b mRNA. Several in vivo experiments also have demonstrated that progesterone induces expression of P-GP mRNA and protein (Arceci et al. 1988; Arceci et al. 1990). In a placental cell line (JAR cell) and a P-GP overexpressing cell line, progesterone and β-estradiol also significantly increased levels of P-GP, decreasing uptake of P-GP substrates saquinavir and paclitaxel (Coles et al. 2009b). Other sex-steroid hormones estrone, estradiol, and ethinyl estradiol were reported to induce expression of P-GP and mRNA (Kim and Benet 2004). Several studies demonstrated that hat hypoxia significantly increased levels of P-GP mRNA and protein (Javam et al. 2014; Lye et al. 2013). These results suggest that changes in oxygenation of the placenta may alter levels of placental P-GP, which partly explains the fact that high expression of placental P-GP occurs in early gestation and then decreases along with gestation.

Exposure of the fetus and placenta to maternal glucocorticoids is normally limited by the placental glucocorticoid barrier. P-GP, serving a components of placental glucocorticoid barrier, mediates efflux of cortisol from cells (Farrell et al. 2002). In BeWo and BeWoMDR (BeWo cells virally transduced with P-GP), it was found that the overexpression of P-GP in BeWoMDR cells remarkably reduced activation of the glucocorticoid receptor by dexamethasone and cortisol to about 40% of those in BeWo cells. Coadministration of cyclosporine A almost reversed glucocorticoid receptor activation in BeWoMDR cells to levels in BeWo cells. Moreover, transport of dexamethasone across BeWoMDR monolayers was lower than that across BeWo monolayers, and the difference was eliminated by cyclosporine A (Farrell et al. 2002). These data indicate that P-GP also contributes to the placental glucocorticoid barrier and that 11β-hydroxysteroid dehydrogenase type 2 and P-GP may act in unison to regulated fetal and placental exposure to glucocorticoids. Glucocorticoids

also mediate placental P-GP expression. In human first-trimester placental explants (7–10 weeks), it was found that 48-h treatment of dexamethasone and cortisol significantly increased P-GP mRNA levels but not P-GP (Lye et al. 2018). Dexamethasone and betamethasone but not prednisone significantly induced expression of P-GP mRNA by around fourfold. In parallel, 100 nM betamethasone decreased the glucocorticoid receptor gene expression by 22% (Manceau et al. 2012).

Pregnant FVB mice were treated with dexamethasone (0.1 mg/kg or 1 mg/kg; s.c.) or vehicle (saline) from either midgestation (embryonic day 9.5–15.5) or late gestation (embryonic day 12.5–18.5) for 6 days. On the last day (embryonic day 15.5 or embryonic day 18.5), function and expression of placental P-GP were assessed. The results showed that dexamethasone (1 mg/kg) treatment in late gestation significantly upregulated expression of Abcb1a mRNA and P-GP protein. Dexamethasone (1 mg/kg) treatment in midgestation also significantly increased expression of placental Abcb1a mRNA (Petropoulos et al. 2010). Regulation of P-GP expression by dexamethasone seemed to be dependent on physiological status. A report showed that expressions of both Abcb1a and Abcb1b mRNA in the labyrinth zone were markedly higher than those in the junctional zone of rat placenta (Mark et al. 2009). Abcb1b and Abcb1a are expressed predominantly in the labyrinth zone and are thus ideally placed to limit the transfer of endogenous glucocorticoids and exogenous xenobiotic compounds from the maternal to the fetal circulation. Compared with preterm human placentas without chorioamnionitis, preterm human placentas with chorioamnionitis showed significant decreases in P-GP staining by immunohistochemistry, which was contrast to expression of P-GP mRNA (do Imperio et al. 2018). Pregnant ICR mice were injected intraperitoneally with different doses of lipopolysaccharides (LPS) (0.1–0.5 mg/kg) on gestational day 17. Results showed that LPS significantly downregulated pregnane X receptor (PXR), cyp3a11, and Abcb1a mRNA levels in a dose-dependent manner, accompanied by increases in lipid peroxidation and pro-inflammatory cytokine expressions in mouse placenta. Alpha-phenyl-N-t-butylnitrone and N-acetylcysteine significantly attenuated LPS-induced alterations, indicating that LPS downregulate placental Pxr, cyp3a11, and Abcb1a mRNA expressions via activating reactive oxygen species pathways (Chen et al. 2005). Similarly, single intraperitoneal doses of polyinosinic:polycytidylic acid [poly(I:C)] (2.5 or 5.0 mg/kg) to regnant rats significantly downregulated placental Abcb1a/Abcb1b, Abcc1, Abcc3, Abcg2, Slco1a4, and Slco4a1 mRNA, accompanied by significant increases in plasma concentrations of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin (IL)-6 (Petrovic and Piquette-Miller 2010). In consistence with decreases in P-GP expression, a report showed that higher levels of accumulation, when normalized to unbound lopinavir, were seen in the fetus and placenta of poly(I:C)-treated rats (Petrovic and Piquette-Miller 2015).

P-GP is also expressed in the proliferating cytotrophoblast, the syncytiotrophoblast, and the extravillous trophoblast, acting as a molecular sieve. A report showed that

silencing of *ABCB1* gene via siRNA duplex dramatically reduced invasion and migration and increased tube formation and fusion in HTR8/SVneo cell line (extravillous trophoblast-like). In both extravillous trophoblast and cytotrophoblast explant differentiation experiments, it was found that silencing of *ABCB1* gene induced expressions of the fusion markers (such as human chorionic gonadotropin, syncytin-1, and gap junction alpha-1 protein), and led to terminal differentiation of both trophoblast subtypes. Moreover, P-GP levels were decreased in both the villous and the extravillous trophoblast of severe early-onset preeclamptic placentas. These results indicate that, in addition to its role as a syncytial transporter, P-GP is a key factor in the maintenance of both cytotrophoblast and extravillous trophoblast lineages and that its decrease in severe preeclampsia may contribute to the syncytial and extravillous trophoblast placental pathologies associated with this disease (Dunk et al. 2018).

Assessment of placental P-GP activity will aid to choice of therapeutic drugs in pregnancy. An example of optimization of drug therapy in pregnancy is ivermectin (Brown 1998). This drug, a P-GP substrate, crosses the placenta only in a negligible amount, indicating that the drug may be used in the treatment of onchocerciasis in pregnant women. Another important example relevant to the role of placental P-GP is the risk of P-GP-mediated drug-drug interactions (DDIs). Some cardiovascular agents such as verapamil and quinidine are substrates and inhibitors of P-GP. They may induce DDIs with digoxin (Lin 2003). Since digoxin is a drug commonly used to treat fetal tachycardia via maternal drug administration (Kleinman and Nehgme 2004; Oudijk et al. 2002), DDIs may increase fetal plasma levels and adverse effects. Although there are no clinical reports demonstrating interactions with placental P-GP so far, based on the current knowledge of functional activity of placental P-GP, potential risk of P-GP-mediated DDIs should be carefully assessed when a potent inhibitor and substrate of P-GP are administered concomitantly to the pregnant women. In some cases, to increase drug penetration to the fetus via inhibiting placental activity and expression of placental P-GP would be beneficial. For example, sustained fetal tachyarrhythmia is a potentially life-threatening condition for the unborn. Digoxin is commonly used as an initial monotherapy. Flecainide, sotalol, and verapamil are also used as a monotherapy or a combination therapy with digoxin. The treatment success rate with digoxin is only about 50% due to poor placental transfer of digoxin. A challenging task is how to maximize fetal drug exposure, while minimizing drug exposure of the mother. A beneficial suggestion is to enhance digoxin availability to the fetus via inhibiting of P-GP (Ito 2001). Another example is how to improve pharmacotherapy of the unborn child when pregnant women infected by HIV receive antiretroviral therapy. Highly active antiretroviral therapy including HIV protease inhibitors is used to reduce the rate of mother-to-child HIV transmission (Capparelli et al. 2005). However, these protease inhibitors are often substrates of P-GP, which do not cross the placenta in an appreciable amount and cannot be expected to exert a direct activity in utero (Marzolini et al. 2002). Importantly, compared with placentas of healthy pregnant women, placentas of HIV-infected women also showed higher expression of P-GP (Camus et al. 2006), in turn, further strengthening this therapeutic obstacle. To

increase materno-fetal penetration of antiretrovirals via P-GP inhibitors may be advantageous selection (Huisman et al. 2000; Clayette et al. 2000).

11.3 Breast Cancer Resistance Protein (BCRP/ABCG2) and Blood-Placental Barrier

BCRP, coded by *ABCG2* gene, is highly expressed in the placenta of humans and rodents. Immunostaining analysis have demonstrated that BCRP is primarily localized on the apical side of the syncytiotrophoblasts and fetal capillary endothelial cells in human (Lye et al. 2018; Yeboah et al. 2006). BCRP is also expressed in the human amniotic epithelium, chorion, and decidua (Aye et al. 2007). Further analysis revealed that BCRP is co-localized with lipid raft proteins in detergent-resistant, lipid raft-containing fractions from the placental microvillous membranes (Szilagyi et al. 2017). In rodents, Bcrp is also mainly expressed in the syncytiotrophoblast layers in the labyrinth and some vascular structures within the myometrium (Staud et al. 2006; Jonker et al. 2002; Petrovic et al. 2008). Immunostaining analysis showed that Bcrp is mainly located on the apical membrane of the placental syncytiotrophoblast II layer, but not the syncytiotrophoblast I layer in rodents (Akashi et al. 2016). Likely to P-GP, placental BCRP acts as gatekeepers for fetal exposure through excreting toxins and drugs back into the maternal circulation, as evidenced by higher fetal transfer of substrates in *Abcg2*$^{-/-}$ mice and by the high fetal-to-maternal per maternal-to-fetal clearance ratios of substrates. It was reported that although maternal plasma area under the concentration-time curve (*AUC*) of nitrofurantoin in the *Abcg2*$^{-/-}$ pregnant mice was only slightly (but significantly) higher than that in the wild-type pregnant mice (about 1.24-fold of wild-type pregnant mice), the fetal *AUC* of nitrofurantoin in the *Abcg2*$^{-/-}$ pregnant mice was approximately fivefold greater than that in the wild-type pregnant mice (Zhang et al. 2007). Compared with wild-type mice, the free concentrations of zearalenone and its two metabolites α-zearalenol and β-zearalenol in fetal of *Abgc2*$^{-/-}$ mice were increased by 115%, 84%, and 150% of wild-type mice, respectively. Concentrations of free zearalenone and α-zearalenol in *Abcg2*$^{-/-}$ placentas were elevated to 245% and 178% of wild-type pregnant mice (Szilagyi et al. 2018), respectively. Jonker et al. (2000) reported that fetal penetration of topotecan (BCRP substrate) in P-GP-deficient pregnant mice treated with elacridar was 2-fold higher than that in P-GP-deficient pregnant mice without elacridar. BCRP mediates transport of glyburide from the fetal to the maternal circulation. It was found that the fetal AUC of glyburide in the *Abcg2*$^{-/-}$ pregnant mice was approximately two times greater than that in the wild-type pregnant mice, although plasma AUCs of glyburide in *Abcg2*$^{-/-}$ and in the wild-type pregnant mice were comparable (Zhou et al. 2008). These results suggest that BCRP has an important protective function at the maternal-fetal interface similarly to P-GP. In vitro placental perfusions are often used to assess drug transport across placentas. In the rat placental

perfusion study, it was found that fetal-to-maternal clearance of cimetidine was found to be 25-fold higher than that in the opposite direction and that BCRP inhibitors fumitremorgin C (2 μM) or elacridar (2 μM) partly eliminated the divergence (Staud et al. 2006). In vitro perfused term human placenta experiments also demonstrated that transports of 2-amino-1-methyl- 6-phenylimidazo[4,5-b]pyridine (PhIP) (Myllynen et al. 2008) and glyburide (Pollex et al. 2008) from maternal to fetal circulation were enhanced by BCRP inhibitors. Moreover, the expression of BCRP in perfused tissue was negatively correlated with the fetal-to-maternal ratio of PhIP (Myllynen et al. 2008). A report (Evseenko et al. 2007) demonstrated important roles of BCRP in protection of the human placental trophoblasts from apoptosis and contributions of decreases in BCRP expression to idiopathic fetal growth restriction. BCRP inhibitor Ko143 augmented cytokine (TNF-α/IFN-γ)-induced apoptosis and phosphatidylserine externalization in primary trophoblast and BeWo cells. Silencing of BCRP expression in BeWo cells significantly increased their sensitivity to apoptotic injury in response to cytokines and exogenous C6 and C8 ceramides, accompanied by significant increases in intracellular ceramide levels after cytokine exposure. BCRP expression in placentas from pregnancies complicated by idiopathic fetal growth restriction was also significantly lower than that in placentas from normal pregnancies (Evseenko et al. 2007).

Placental BCRP is able to transport bile acids, indicating that BCRP may play a key role in bile acid transport in the placenta, as bile salt export pump (BSEP) does in the liver (Blazquez et al. 2012). In Chinese hamster ovary coexpressed rat Oatp1a1 and human ABCG2, it was found that coexpression of two transporters enhanced the uptake and efflux, respectively, of cholylglycylamido fluorescein, cholic acid, glycocholic acid, taurocholic acid (TCA), and taurolithocholic acid-3-sulfate (Blazquez et al. 2012). Obstructive cholestasis in pregnant rats only slightly increased fetal cholanemia, less than twofold, although maternal cholanemia significantly increased more than 14-fold. The expression of Bcrp in rat placenta was much higher than that of Bsep. In pregnant rats, fumitremorgin C did not affect uptake/secretion of glycocholic acid by the liver but inhibited fetal-maternal transfer of glycocholic acid. Compared with wild-type mice, obstructive cholestasis in pregnant *Abcg2*$^{-/-}$ mice induced similar bile acid accumulation in maternal serum but significantly increased accumulation of bile acids in the placenta, fetal serum, and liver (Blazquez et al. 2012). Acetaminophen treatment also downregulated expression of placental Bcrp in rats, which was related to drug-induced enhanced oxidative stress in trophoblast cells. In line with decreased placental Bcrp expression, in situ perfused rat placenta demonstrated reduction in the Bcrp-dependent fetal-to-maternal bile acid transport (Blazquez et al. 2014). In human, it was reported that expressions of BCRP and P-GP were significantly increased in the intrahepatic cholestasis of pregnancy (ICP) compared with normal pregnancy. The increases in expression of BCRP were further enhanced by ursodeoxycholic acid (UDCA) (Estiú et al. 2015). Another report showed that ICP induced 1.5-fold increases in expression of BCRP compared with normal placenta and that high dose of UDCA (25 mg/kg/day) further enhanced expression of BCRP mRNA and protein to 4.2-fold and 2.8-fold of the placenta without UDCA treatment (Azzaroli et al. 2013),

respectively. These results indicated that the enhanced expression of placental BCRP in ICP could be responsible for the maintenance of low bile acid concentrations in the placenta and, subsequently, in the fetal body, despite the marked increase in these compounds in the maternal circulation, demonstrating an important roles of BCRP in the placental transport of bile acids (Blazquez et al. 2012).

The pattern of placental BCRP expression over gestation has been found to be inconsistent among various studies. An analysis from isolated human placentas of various gestational ages (6–13 weeks, 16–19 weeks, 24–29 weeks, 32–35 weeks, 38–41 weeks of gestation, before and after labor) showed that BCRP mRNA was highly expressed in all tested tissues without statistic differences (Yeboah et al. 2006). However, the expressions of BCRP mRNA did not match with the expression of BCRP. The term placenta showed higher expression of BCRP than those in other preterm placentas (Yeboah et al. 2006) although labor did not affect levels of BCRP (Yeboah et al. 2006; Yeboah et al. 2008). The numbers of fetal blood vessels immunostained were also higher in term than in tissue of 6–13 week gestation (Yeboah et al. 2006). However, a report showed that expression of placental BCRP (protein and mRNA) did not change significantly with gestational age based on human placentas of several gestational ages (60–90 days, 90–120 days, and full-term C-section placentas) (Mathias et al. 2005). In contrast, several reports showed that expression of BCRP significantly decreased with advancing gestation (Meyer zu Schwabedissen et al. 2006; Petrovic et al. 2015; Williams et al. 2012). In pregnant rat, Bcrp mRNA expression was detected in all placentas of each examined gestation day 12, 15, 18, and 21. The expression of Bcrp mRNA peaked on gestation day 15 and declined significantly to one third up to term. In consistence, the amount of cimetidine in the fetus after the infusion to dams reached peak on gestation day 12, and the ratio of fetal-to-maternal cimetidine concentrations (1.18) was almost ten times higher for fetuses of gestation day 12 in comparison with fetuses of the 15th day (0.105), 18th day (0.135), and 21st day (0.120), indicating poor protection of fetus against cimetidine at the very early stage of pregnancy (Cygalova et al. 2008). Consistently, another study showed that the levels of Bcrp mRNA and protein in the rat placenta on gestation day 20 were lower than those on gestation day 14 (Yasuda et al. 2005). In the placenta of pregnant mice on embryonic day 9.5, 12.5, 15.5, and 18.5, it was found that expression of Bcrp mRNA peaked within the placenta on embryonic day 9.5 and significantly decreased thereafter. However, expression of Bcrp did not change significantly during gestation (Kalabis et al. 2007). Similar report showed that expression of Bcrp (mRNA and protein) in mouse placenta peaks on gestation day 15 compared to gestation days 10 and 19 (Wang et al. 2006a).

Numerous studies have investigated the mechanisms and factors that affect expression of placental BCRP. In BeWo cells, it was found that pregnancy-related hormones estradiol, human placental lactogen, and human prolactin at physiological concentrations significantly increased BCRP and mRNA approximately two- to threefold. Induction of BCRP by estradiol was abrogated by the estrogen receptor antagonist ICI-182,780 but not knockdown of estrogen receptor α by RNA interference. Human chorionic gonadotropin at physiological concentrations had no effect on BCRP expression (Wang et al. 2008). Progesterone and 17β-estradiol

significantly increased and decreased BCRP protein and mRNA, respectively. Estrogen receptor antagonist ICI-182,780 may abrogate 17β-estradiol-induced reduction in BCRP expression, but the progesterone receptor antagonist RU-486 little affect progesterone-mediated induction of BCRP (Wang et al. 2006b). Epidermal growth factor significantly increased expression of BCRP mRNA and protein in cytotrophoblasts, BeWo, and MCF-7 cells (Meyer zu Schwabedissen et al. 2006). In Jar cells and human primary trophoblasts, it was found that the 24-h treatment with PGE2 significantly also upregulated BCRP expression, leading to decreases in cellular accumulation of the fluorescent substrate Hoechst 33342. E-prostanoid receptor antagonists attenuated the induction of BCRP expression by PGE2, demonstrating roles of E-prostanoid receptor signaling in BCRP expression (Mason et al. 2014). Moreover, peroxisome proliferator-activated receptor gamma (PPARγ) agonist rosiglitazone enhances expression of BCRP mRNA, resulting in a 20% greater efflux of the substrate Hoechst 33342 from BeWo cells compared with control cells (Lin et al. 2017).

Women who gave birth at a high altitude exhibited signs of chronic placental hypoxia. Hypoxia decreased expression of the BCRP mRNA and protein via increasing hypoxia inducible factor-1α protein signaling (Francois et al. 2017). Inflammation may affect expression of placental BCRP. A report showed that greater expression of P-GP and BCRP was observed in placentas of women with preterm labor than in placentas of those with term labor. Moreover, expression of BCRP and P-GP (mRNA and protein) also increased in placentas of women with preterm labor with inflammation. Levels of BCRP and P-GP mRNA were correlated with that of IL-8, which also increased significantly in preterm labor with inflammation samples (Mason et al. 2011). Increased expressions of BCRP (protein and mRNA) were found in preterm human placentas with chorioamnionitis. The increased expression of BCRP and IL-8 mRNA were significantly correlated to the severity of chorioamnionitis (do Imperio et al. 2018). Exposure to LPS and polyinosinic:polycytidylic acid (a viral antigen) decreased BCRP mRNA and protein expression in human placental explants from the first and third trimesters, respectively (Lye et al. 2015).

In pregnant rats, it was found that treatment of endotoxin significantly downregulated expressions of Bcrp protein and mRNA. Pharmacokinetics data showed that although administration of endotoxin resulted in comparable plasma concentrations of glyburide, the accumulation of glyburide in the fetuses of endotoxin-treated rats pronouncedly increased to 162% of control rats (Petrovic et al. 2008). Human placental samples from preterm and term pregnancies diagnosed with chorioamnionitis infection showed that expressions of placental BCRP mRNA and protein were downregulated by about 50% of healthy control, accompanied by significant decreases in mRNA levels of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α. These data show that maternal infections impact the expression of key drug transporters in the placenta (Petrovic et al. 2015), suggesting that bacterial infection may alter exposure of the conceptus to toxins and drugs in early pregnancy, whereas viral infection may disrupt fetal protection in later gestation. Moreover, preeclampsia may alter expression of placental BCRP. It was reported that compared to placentas from healthy pregnancies, preeclamptic placentas

had increased number of syncytial knots with increased expression of BCRP in their apical membrane (Afrouzian et al. 2018).

Glucocorticoids also regulate expression of placental BCRP. It was reported that dexamethasone and cortisol increased expression of BCRP mRNA and protein in first-trimester placental explants. Moreover, the induction of BCRP by dexamethasone was dependent on phenotypes in BeWo cells. Dexamethasone increased expression of BCRP in syncytiotrophoblast phenotype (syncytialized states) but not cytotrophoblast phenotype (non-syncytialized states) (Lye et al. 2018). A report showed that expression of placental BCRP increased with trophoblast differentiation (Evseenko et al. 2006), which was in line with high expression of BCRP in the syncytiotrophoblast cells. The upregulation was further enhanced following exposure to dexamethasone, suggesting that syncytiotrophoblast cells are the response site for glucocorticoid-mediated BCRP upregulation in the placenta and that glucocorticoids may represent an important inducer of development and differentiation of the trophoblast protective barrier throughout pregnancy or, at least, during the first trimester of gestation. In contrast, a report showed that pregnant mice from embryonic day 9.5 to 15.5 or embryonic day 12.5 to 18.5 were treated with dexamethasone (1 mg/kg) for 6 days. Significant downregulation of Bcrp mRNA expression and significant downregulation of Bcrp function occurred on embryonic day 15.5 (Petropoulos et al. 2011). These results indicate that data obtained from animal studies could possibly not be directly translatable to humans.

11.4 Multidrug Resistance-Associated Proteins (MRPs/ABCCs) and Blood-Placental Barrier

MRPs, coded by *ABCC* genes, consist of nine members. The best studied isoforms of MRPs in human and murine placenta are main MRP1, MRP2, MRP3, MRP4, and MRP5 (Joshi et al. 2016; Behravan and Piquette-Miller 2007).

11.4.1 MRP1/ABCC1 and Blood-Placental Barrier

MRP1, a 190 kDa protein encoded by the *ABCC1* gene, is most abundant within terminal villi at the basolateral surface of fetal blood vessel endothelial cells and syncytiotrophoblast cells with some evidence for expression in the apical syncytiotrophoblast (Atkinson et al. 2003; Maher et al. 2005; Nagashige et al. 2003; St-Pierre et al. 2000). Characteristics of 2,4-dinitrophenyl-S-glutathione transport in the placental villous tissue also demonstrated existence of MRP1 in the human placental syncytiotrophoblast (Vaidya et al. 2009). MRP1 protein can be detected in primary cultures of cytotrophoblast cells, in purified basal membranes

isolated from the syncytiotrophoblast of the term placenta, in BeWo cells, and in Jar cells (Evseenko et al. 2006; Nagashige et al. 2003; Pascolo et al. 2003), but MRP1 protein was present in much lower amounts in primary trophoblasts compared with BeWo cells and Jar cells (Evseenko et al. 2006). This basolateral localization of MRP1 at syncytiotrophoblast is believed to export substrates toward the fetal circulation (Atkinson et al. 2003; Nagashige et al. 2003), whereas the abluminal expression within fetal endothelial cells is directed away from the fetal circulation toward the syncytiotrophoblast (Nagashige et al. 2003). The two localizations seem to be at cross purposes, but their relative abundances suggest that the clearance of its substrates from the fetal circulation precedes over import from the syncytiotrophoblast toward the fetus (St-Pierre et al. 2000).

Expression of placental MRP1 increases with gestation. It was reported that expression of Mrp1 protein increased 26-fold in the placenta of C57BL/6 mice from gestation day 14 to 17 (Aleksunes et al. 2008). Another report (Coles et al. 2009a) showed that Mrp1 expression in the placenta of CD-1 mice was significantly greater (130%) at late gestation (gestation day 18) compared to midgestation (gestation day 13). Similarly, in human, the levels of MRP1 mRNA in full-term placenta were 4-fold higher than those in the early gestational age (9–10 weeks) and increased 20-fold with polarization of BeWo cells (Pascolo et al. 2003).

Roles of placental MRP1 have been demonstrated in vivo and in vitro. MRP1 mediates unconjugated bilirubin transport, protecting from its cellular toxicity. Data from the human trophoblastic cells and BeWo cells showed that unconjugated bilirubin efflux from cells was inhibited by MRP inhibitors sulfobromophthalein and MK571 (Pascolo et al. 2001). Similarly, uptake of unconjugated bilirubin by apical plasma membrane vesicles from human placental trophoblast at term was activated by ATP hydrolysis but inhibited by vanadate and doxorubicin, cholic acid, methotrexate, and probenecid (Serrano et al. 2002). These results infer roles of MRP1 in transport of unconjugated bilirubin (Pascolo et al. 2001; Serrano et al. 2002). In contrast, uptake of unconjugated bilirubin by basal plasma membrane vesicles was independent of ATP hydrolysis and cis-inhibited by estrone-3-sulfate (E3S) and estradiol-17 beta-d-glucuronide ($E_2$17βG) (Serrano et al. 2002). A report demonstrated that OATP1B3 mediates uptake of unconjugated bilirubin by basal plasma membrane (Briz et al. 2003a). Thus, export of bilirubin from fetal endothelial cells would logically precede the subsequent steps: i.e., OATP1B3 mediated uptake at the basal surface of the syncytiotrophoblast, and then MRP1 mediates efflux into maternal blood at the apical side (Briz et al. 2003b). Monkey experiment (Sikkel et al. 2004) demonstrated that compared with normal rhesus, fetuses of alloimmunized anemic rhesus showed higher fetal blood bilirubin concentrations throughout the course of gestation than normal rhesus. The amniotic fluid/fetal blood ratio for bilirubin in alloimmunized anemic fetuses decreased from 0.09 at 28 weeks to 0.05 at 33 weeks. Amniotic fluid/fetal blood ratios for bilirubin in normal fetuses also showed decrease with gestation. These results may be explained by the findings that expression of MRP1 increased fourfold in the third as compared with first-trimester placenta (Pascolo et al. 2003) and that increased bilirubin production in phenylhydrazine-treated rats was correlated with an upregulation of Mrp1 expression in the liver and spleen (Cekic et al. 2003).

Another role of placental MRP1 seems to defense against oxidative stress via exporting glutathione disulfide (GSSG) and some peroxidation products (such as 4-hydroxy-2-nonenal). Indeed, accumulation of 4-hydroxy-2-nonenal, the major lipid peroxidation product, could be detected within villous endothelium in an experimental model of oxidative stress (Hung et al. 2001) and in the placentas from preeclamptic women (Chekir et al. 2006; Hnat et al. 2005; Shibata et al. 2001). Both GSSG and the glutathione-S conjugate of 4-hydroxy-2-nonenal are substrates of MRP1. Moreover, the increased oxidative stress in turn upregulates expression of MRP1 (Tatebe et al. 2002; Yamane et al. 1998), supporting the notion that MRP1 protects fetal endothelial cells by exporting potentially noxious products. cAMP, as a second messenger, exerts an important role in the control of human first-trimester trophoblast functions. MRP1 was reported to extrude cAMP from trophoblast-derived cell lines, trophoblasts, and human first-trimester placenta, inferring that placental MRP1 is implicated in the control of syncytiotrophoblast secretory activity and extravillous trophoblast, cell proliferation, migration, and invasiveness (Biondi et al. 2010). In addition, in vitro data also showed that MRP1 may be involved in mercury kinetics of trophoblast cells (Straka et al. 2016).

11.4.2 MRP2/ABCC2 and Blood-Placental Barrier

MRP2, encoded by the *ABCC2* gene, shares similar substrate selectivity with MRP1, but its tissue distribution substantially differs from MRP1. The patterns of MRP2 expression and localization is more similar to those of P-GP and BCRP. MRP2 is mainly expressed in the apical membrane of placental syncytiotrophoblasts. Although MRP2 mRNA are highly expressed in BeWo cells, Jar cells and primarily cultured trophoblasts, but MRP2 protein was present only at very low levels in cultured trophoblasts and was practically undetectable in BeWo and Jar cells (Evseenko et al. 2006). In mice, expression of Mrp2 was higher in fetal membranes than the placenta. Mrp2 protein expression in fetal membranes was five- and twofold higher than in the placenta at days 14 and 17 of gestation. Consistently, expression of Mrp2 mRNA in fetal membranes was seven- and eightfold higher than in the placenta at days 14 and 17 of gestation (Aleksunes et al. 2008). Several reports have demonstrated that expression of placental MRP2 is dependent on gestational age with conflicting results. For example, Meyer zu Schwabedissen et al. (2005a) compared expression of MRP2 mRNA and proteins in early preterm (<32 weeks), in late preterm (32–37 weeks), and in term (>37 weeks) placentas. They found that expressions of MRP2 significantly increased with gestational ages. Levels of MRP2 mRNA in late preterms and in terms were increased to 2.74-fold and 4.88-fold of that in early preterm, respectively. Similarly, levels of MRP2 protein in late preterms and in terms were increased to 1.25-fold and 1.64-fold of that in early preterm, respectively. In cultured cytotrophoblasts, MRP2 expression increased with differentiation to syncytiotrophoblasts, with a peak on day 2, inducing increases by 13.7-fold (Meyer zu Schwabedissen et al. 2005a). Similar report showed that expression of

MRP2 mRNA in the placenta of late gestation significantly increased to twofold of that in early gestational stages (Pascolo et al. 2003). But Williams et al. (2012) reported that expression of MRP2 protein significantly decreased with gestational ages. They found that levels of MRP2 protein in early second trimester (mean 12.9 weeks) and in third trimester (mean 39.4 weeks) were only 47.7% and 16.5% of those in first trimester (mean 8.8 weeks). However, expressions of Mrp2 protein in mouse placenta from gestation day 14 to 17 were comparable (Aleksunes et al. 2008).

MRP2 substrate selectivity differs markedly from P-GP and BCRP. MRP2 prefers anionic substrates, which are generally made somewhat more hydrophilic through formation of glucuronide, glutathione, and sulfate conjugates. Functions of MRP2 in placental syncytiotrophoblasts are to provide fetal protection against harmful drugs/xenobiotics and endogenous toxic substances via effluxing these compounds from fetus into maternal circulation. In human cotyledon perfusion, it was found that clearance indexes of a CCR5 antagonist maraviroc across the placenta in fetal-to-maternal direction were twofold of that in maternal-to-fetal direction. A significant inverse correlation between maraviroc clearances in the maternal-to-fetal direction and levels of placental MRP2, ABCC10 and ABCC11 mRNA was observed (Vinot et al. 2013). Similarly, human placenta perfusion demonstrated that the mater-to-fetal transfer of talinolol was significantly lower than creatinine. Maternal-to-fetal transfer of talinolol was significantly increased by the MRP2 inhibitor probenecid and the nonspecific inhibitor verapamil (May et al. 2008). These results indicate that maternal-to-fetal transfers of talinolol and maraviroc are also restricted by MRPs (Vinot et al. 2013; May et al. 2008). MRP2 may be involved in the extraction of mercuric ions from placental and fetal tissues. It was reported that the amount of mercury in fetus tissues (renal, liver), fetus blood, amniotic fluid, uterus, placentas, and whole fetuses from pregnant TR^{-} rats (Mrp2-deficient) was significantly greater than that from pregnant Wistar rats following exposure to a single dose of methylmercury (Bridges et al. 2012).

The placenta lacks expression of BSEP; thus MRP2 may be implicated in the excretion of bile salt conjugates synthesized by the maturing fetal liver (St-Pierre et al. 2000). Interestingly, Mrp2 expression in rat placenta increased during obstructive cholestasis and furthermore upon treatment with UDCA (Serrano et al. 2003). Clinical trial also showed that women with ICP, ursodeoxycholic treatment increased placental MRP2 expression (mRNA and protein) and reduced bilirubin and bile acid levels in cord blood, but did not affect expression of MRP3 and MRP4 (Azzaroli et al. 2007). Likely to MRP1, MRP2 also mediates transport of bilirubin and conjugated bile acids, indicating that MRP2 may be involved in the transfer of such endogenous compounds synthesized by the fetal liver, across the placental syncytiotrophoblast (Marín et al. 2005; 2008), demonstrating the role of MRP2 in placental excretion of bile salt conjugates. The placenta also shows the expression and activity of UDP-glucuronosyltransferases isoforms 2B4 and 2B7. These enzymes catalyze formation of glucuronide conjugates, which could be efficiently excreted by MRP2, detoxificating exogenous and endogenous ligands and

maintaining placental function through clearance and regulating steroid hormone homeostasis (Collier et al. 2002). Similarly, zidovudine in the placenta may be metabolized to zidovudine-glucuronide and then is preferentially excreted into the maternal circulation (Collier et al. 2004). It should be noted that many transporters such as MRP1, MRP2, and MRP3 may be involved in secretion of zidovudine-glucuronide in the placenta. MRP2 is positioned in the apical syncytiotrophoblast, while MRP1 and MRP3 are located in the fetal capillary. They work in series to accomplish the secretion of zidovudine-glucuronide into maternal blood.

Inflammation may also affect expression of placental MRP2 with contradictory. do Imperio et al. (2018) reported that chorioamnionitis increased expression of MRP2 mRNA in preterm human placentas, which was positively correlated to the severity of chorioamnionitis. But in rats, it was reported that placental expression of Mrp1, Mrp2, and Mrp4 was not significantly affected by bacterial endotoxin (Ghoneim et al. 2017). In contrast, Petrovic et al. (2008) reported that endotoxin treatment downregulated mRNA levels of Abcb1a/Abcb1b, Mrp1, Mrp2, and Mrp3 in rat placenta.

11.4.3 MRP3/ABCC3 and Blood-Placental Barrier

MRP3, coded by *ABCC3* gene, is predominantly expressed in the fetal blood vessel endothelia and shows minor expression in the apical syncytiotrophoblast (St-Pierre et al. 2000). Expression of MRP3 mRNA in trophoblasts is abundant, and its expression in the trophoblast-derived cell line BeWo, JAR, and JEG-3 is very weak (Serrano et al. 2007). The expression of MRP3 mRNA in the placenta was almost 10,000 times greater than BeWo cells. Moreover, MRP3 expression in BeWo cells was upregulated by polarization (Serrano et al. 2007). Unlike MRP1 and MRP2, expressions of MRP3 in first- and third-trimester placenta are comparable (Pascolo et al. 2003). MRP3 expression in the placenta shows great species differences. Mrp3 is barely detectable in the mouse placenta (Maher et al. 2005). In the rat placenta, Mrp3 is predominantly localized on the apical membranes of the endodermal epithelial cells that line the yolk sac diverticula and to a lesser extent in the endothelium of the maternal arteries and the syncytiotrophoblast layers of the labyrinth zone and endothelium of the maternal feeder arteries, but not in endothelial cells of the fetal capillaries (St-Pierre et al. 2004). Strong expression of Mrp3 in the epithelial cells surrounding the yolk sac diverticula may suggest that Mrp3-mediated efflux into the yolk sac is an excretory pathway in the rat placenta. MRP3 substrate specificity resembles MRP1 and MRP2, mediating the efflux of several organic anions including their sulfates and glucuronide conjugates. Likely to MRP1, the preferential subcellular localization of MRP3 in fetal blood vessel endothelia protects fetus from toxic substances via efflux of the toxic substrates in the fetal-to-maternal direction. Rat Mrp3 was reported to accept bile salts (such as taurocholate, glycocholate, taurochenodeoxycholate-3-sulfate, and taurolithocholate-3-sulfate) as

substrates (Hirohashi et al. 2000; Akita et al. 2002). Human MRP3 also mediates transport of glycocholate and taurolithocholate-3-sulfate (Akita et al. 2002). Moreover, Mrp3 expression is upregulated during maternal cholestasis and upon treatment with UDCA, suggesting a defensive role for Mrp3 in limiting fetal exposure to elevated levels of maternal bile acids (Serrano et al. 2003). However, findings in rats were not repeated in patients. In patients with ICP, it was found that treatment with ursodeoxycholate remarkably increased mRNA expression in placentas without altering protein expression (Azzaroli et al. 2007). Furthermore, MRP3 expression in the placenta may be associated with low distribution of some xenobiotics in the fetus. Glyburide is substrate of MRP3 and BCRP, indicating that MRP3 mediates transplacental transfer of glyburide to the fetus as does BCRP (Gedeon et al. 2006), which may become a reason leading to very low transplacental transport of glyburide to the fetal circulation (Kimber-Trojnar et al. 2008). Additionally, MRP3 may also be involved in efflux of some anti-cancer agents such as cisplatin (Pawłowski et al. 2013) and methotrexate (Kool et al. 1999), which may partly explain why transports of cisplatin (Al-Saleh et al. 2008) or methotrexate (Al-Saleh et al. 2007) in maternal-to-fetal direction are lower compared with reference antipyrine.

11.4.4 MRP4/ABCC4 and Blood-Placental Barrier

MRP4, coded by *ABCC4* gene, is expressed in apical membranes of human term placental syncytiotrophoblasts (Azzaroli et al. 2007). Unlike MRP2, neither ICP nor ICP patients treated with UDCA affected the expression of MRP4 protein (Azzaroli et al. 2007). Levels of MRP4 mRNA in BeWo, JAR, and JEG-3 cells were higher than in primary human placental trophoblasts (Serrano et al. 2007). Mrp4 mRNA was also detected in the placenta of rats, and rat conditionally immortalized syncytiotrophoblast cell lines TR-TBTs (Lee et al. 2011). In mice, it was found that expression of Mrp4 mRNA in fetal membrane was higher than that in the placenta and that the expression of Mrp4 protein was 136-fold higher in fetal membranes than the placenta at day 14 of gestation. Moreover, levels of Mrp4 protein were markedly reduced by day 17 (Aleksunes et al. 2008). Immunostaining revealed that Mrp4 protein was located on basolateral membrane of yolk sac epithelial cells (Aleksunes et al. 2008). MRP4 mediates the efflux of bile salts, cyclic nucleotides (cAMP and cGMP), urate, steroid conjugates, prostaglandins (such as PGE2), and nucleoside analogs such as adefovir and tenofovir (Slot et al. 2011). In TR-TBTs, it was found that cellar accumulation of 6-mercaptopurine was significantly increased by MRP inhibitors methotrexate, probenecid, cefmetazole, and sulfinpyrazone, indicating that 6-mercaptopurine may be eliminated across the blood-placental barrier via MRPs (Lee et al. 2011). Roles of MRP4 in the human placenta need further investigation.

11.4.5 MRP5/ABCC5 and Blood-Placental Barrier

MRP5, coded by *ABCC5* gene, is a 190 kDa protein, and its expression at the mRNA and protein levels has been reported in the mouse (Aleksunes et al. 2008;Cekic et al. 2003) and human placenta (Meyer zu Schwabedissen et al. 2005b). In preterm placentas the majority of MRP5 is located in the basal membrane and to a minor extent in apical membranes of the syncytiotrophoblasts. MRP5 protein was also detected in the vicinity of fetal blood vessel (Meyer zu Schwabedissen et al. 2005b) and in both apical and basolateral surfaces of the human amniotic epithelium (Aye et al. 2007). MRP5 expression decreases with increase in gestational age. It was reported that levels of MRP5 mRNA in late preterms (32–37 weeks) and in terms (> 37 weeks) were only 72.2% and 15.8% of that in early preterms (<32 weeks), respectively. The expressions of total MRP5 protein in term placentas statistically significantly decreased to 71.6% of that in preterm placentas. Differentiation significantly induced expression of MRP5 mRNA, which was in parallel with the human chorionic gonadotropin production (Meyer zu Schwabedissen et al. 2005b). Similarly, MRP5 mRNA expression in BeWo cells was 1500-fold higher in polarized cells as compared to non-polarized cells, and the expression of mRNA of MRP5 in the placenta of the first trimester was more than 3-fold higher than that in full-term placenta (Pascolo et al. 2003). In line with mRNA content, MRP5 protein was also detected in BeWo cells and placenta tissue, and the expression of MRP5 protein in polarized BeWo cells was particularly higher as compared to non-polarized cells (Pascolo et al. 2003). Mrp5 mRNA was also detected in mouse placenta, significantly lower than in fetal membranes (Pascolo et al. 2003). Mrp5 protein was detected in mouse placenta and fetal membranes at gestation day 14, with reduced expression in both tissues by gestation day 17 (Aleksunes et al. 2008). MRP5 is involved in the ATP-dependent transport of cyclic nucleotide analogs such as 3′,5′-cyclic nucleotides cAMP and cGMP. Trophoblastic vesicles demonstrated ATP-dependent [^{3}H]cGMP transport, but that ATP-dependent [^{3}H]cGMP transport was significantly higher in the basal membrane preparations than in apical membrane preparations, which were in line with MRP5 expression (Meyer zu Schwabedissen et al. 2005b). These results indicate a functional role for MRP5 as an export pump for cyclic nucleotides. cGMP plays an important role in cytotrophoblast differentiation (Meyer zu Schwabedissen et al. 2005b; Sawai et al. 1996), nitric oxide-mediated placental angiogenesis (Yamahara et al. 2003; Xu et al. 2004), and maintenance of fetoplacental vascular tone (Clifton et al. 1995). MRP5 may affect cell differentiation and function via regulating intracellular levels of cyclic nucleotides. Interestingly, in 6-mercaptopurine-treated placental explants, it was found that explants that possessed high expression of MRP5 mRNA did not show migration of extravillous trophoblast cells, whereas all the explants that displayed extravillous trophoblast cell migration (6-mercaptopurine-treated/not treated) had low expression of MRP5 mRNA (Matalon et al. 2008). Besides cGMP-mediated signal transduction, MRP5 may also play a role in the export of organic anions and nucleoside monophosphates of nucleoside analogs used in anticancer and antiviral therapy (Wijnholds et al. 2000).

11.5 Organic Anion-Transporting Polypeptides (OATPs/SLCOs) and Blood-Placental Barrier

Organic anion-transporting polypeptides (OATPs) are SCL family transporters coded by SLCO genes. The identified OATPs in human placenta include OATP1A2 (OATP-A)/SCLO1A2, OATP2B1 (OATP-B)/SLCO2B1, OATP1B3 (OATP-8)/SLCO1B3, OATP3A1 (OATP-D)/SCLO3A1, and OATP4A1 (OATP-E)/SLCO4A1 (Wang et al. 2012; Briz et al. 2003a; Ugele et al. 2003; Patel et al. 2003; Sato et al. 2003). OATP1B1 (OATP-C)/SLCO1B1 mRNA is not or only very lowly expressed (Wang et al. 2012; Patel et al. 2003, Ugele et al. 2003). Both OATP1A2 and OATP1B3 are expressed at mRNA and protein levels in the placenta. OATP1A2 appears to be preferentially localized to basal membrane and apical surface of syncytiotrophoblasts (Wang et al. 2012). OATP1A2 protein is also expressed villous cytotrophoblasts and extravillous trophoblasts (Loubière et al. 2010). OATP1B3 is abundantly expressed at the basal surface of the syncytiotrophoblast throughout gestation and is also localized in cytotrophoblasts before differentiation to the syncytiotrophoblast (St-Pierre et al. 2002; Wang et al. 2012). Similarly, Briz et al. (2003a) reported that abundance of OATP1A2 mRNA in term human placenta and purified mononucleated human trophoblast cells was much higher than that of OATP1B1 but much lower than that of OATP1B3. The abundance of mRNA was OATP1B3> > OATP1A2> > OATP1B1, which was different from the abundance of mRNA in the liver (OATP1B3 > OATP1B1> > OATP1A2) (Briz et al. 2003a).

mRNAs of OATP2B1, OATP3A1, and OATP4A1 were highly expressed in term placental tissue and freshly isolated mononucleated trophoblasts (Ugele et al. 2003). OATP1B3 was intermediately expressed, but OATP1A2 was lowly expressed in term placental tissue and freshly isolated mononucleated trophoblasts. OATP1B1 was only detected at a very low level in term placental tissue but not in isolated and cultured trophoblasts. During cultivation, it was found that expression levels of OATP2B1 decreased after 1 day and increased significantly after 4 days, in parallel with syncytia formation (Ugele et al. 2003). OATP2B1 protein is mainly located at the basal surface of the syncytiotrophoblast and in the cytotrophoblast membranes of human placenta. Interestingly, proliferated trophoblasts (Ki-67 positive) expressed lower levels of OATP2B1 and differentiation induced expression of OATP2B1. In isolated trophoblasts under culture conditions that promoted syncytia formation, it was found that OATP2B1 expression increased eightfold when trophoblasts were differentiated to syncytia (St-Pierre et al. 2002). OATP4A1 protein was preferentially expressed at apical surface of the syncytiotrophoblasts (Loubière et al. 2010; Sato et al. 2003).

Gestational ages seem to also affect expression of placental OATPs. In normal human placenta, it was found that compared to first trimester, expression of OATP1A2 mRNA was downregulated 8-fold and expression of OATP3A1 mRNA was downregulated 17-fold in third-trimester placentas (Patel et al. 2003). Expression of OATP1B1 mRNA was not detected in the third-trimester placenta

(Cui et al. 2009; Patel et al. 2003), while low levels of transcripts were detected in the first-trimester placentas (Patel et al. 2003). Loubière et al. (2010) compared expressions of OATP1A2 and OATP4A1 in placental samples from different gestations (6–10 weeks, 11–14 weeks, 15–20 weeks, 27–34 weeks, and 37–41 weeks). They found that expressions of OATP1A2 and OAP4A1 reached a nadir in midgestation before increasing toward term (Loubière et al. 2010). Similarly, St-Pierre et al. (2004) compared expressions of several Oatp mRNA in mouse placenta of different gestation. They found that Oatp1a1 mRNA was detectable in the placenta at low levels throughout gestation. Oatp1a4 mRNA was not consistently detected in all placenta samples. Oatp1b2 mRNA in the placenta decreased toward the end of gestation. Oatp4a1 mRNA, the most abundant Oatp measured in the placenta, increased throughout gestation (St-Pierre et al. 2004). Maternal bacterial infections affect expression of placental OATP2B1. A clinic report showed that the placental expression of OATP2B1 mRNA in pregnancies with chorioamnionitis infection was significantly decreased to 57 ± 8% of levels in the normal pregnancy (Petrovic et al. 2015). An inverse correlation of OATP2B1 mRNA to expression of IL-1β and IL-6 mRNA was found in preterm placental samples. Consistently, expressions of OATP2B1 protein in preterm pregnancies or term with chorioamnionitis infection were reduced to 68% and 49% of normal values (Petrovic et al. 2015), respectively.

OATP4A1- and OATP2B1-mediated transport in placenta are often coupled to glutamate efflux (Lofthouse et al. 2015, 2018). A report showed that the activity of OATP4A1 on apical membrane of the placental syncytiotrophoblast was coupled to the glutamate gradient. In oocytes expressing OATP4A1, uptake of E3S, thyroid hormones (T3 and T4), and the bile acid taurocholate stimulated glutamate efflux. In term placental villous fragments, addition of E3S and taurocholate trans-stimulated glutamate efflux. Coupling of OATP4A1 to the glutamate gradient may drive placental uptake of E3S, thyroid hormone, and potentially harmful bile acids (Lofthouse et al. 2018). Similarly, cycling of glutamate across the placenta involving efflux via OAT4 and OATP2B1 and subsequent reuptake will drive placental uptake of organic anions from the fetal circulation (Lofthouse et al. 2015).

OATPs mediate transport of some endogenous substances such as bile acids bilirubin, steroids, and thyroid hormones in the placenta. In turn, these endogenous substances also affect expression of placental OATPs. Several reports have demonstrated roles of placental OATPs in transport of bile acids. In BeWo cells, uptake of UDCA from the apical membrane of BeWo cells was time-dependent but sodium-independent and inhibited by inhibitors of energy metabolism and of organic anion transporters, characterizing OATPs (Xia et al. 2018). Ability of OATP1B3 to transport the glycocholic acid and glycochenodeoxycholic acid has been confirmed in trophoblast cell lines SWAN via silencing OATP1B3 (Yan et al. 2015). In BeWo cells, TCA (0.02 mM) significantly induced the mRNA and protein expression of OATP1A2 by 3- and 1.6-fold, respectively. Interestingly, higher concentration of TCA showed weaker induction on OATP1A2. For example, TCA at 0.2 and 2 mM only increased expression of OATP1A2 mRNA and protein by 1.5- and 1.3-fold, respectively. These results indicate that TCA is one of the regulation factors for OATP1A2 in the placenta. Low concentration of TCA can induce fetal membrane

expression of OATP1A2, presenting a physiological or compensatory mechanism of the placenta, but the high concentration of TCA may produce a pathological or pathogenic mechanism (Yang et al. 2014).

ICP, a specific complication in the second half of human pregnancy, is characterized by jaundice, pruritus, elevated serum aminotransferases, and especially elevated bile acids. A clinic report showed that compared with normal human placenta, the placenta of ICP patients showed significantly lower expression of mRNA and protein of both OATP1A2 and OATP1B3 (Wang et al. 2012). OATP1A2 and OATP1B3 expressed at syncytiotrophoblast may play an important role in avoiding accumulation of bile acid in fetus and subsequent toxic effects on fetus. The reduced expression of placental OATP1B3 and OATP1A2 may be responsible for the elevated fetal circulating bile acid and subsequently the increased risk of fetal distress and possible asphyxia. However, another report showed that the intrahepatic cholestasis upregulated expression of OATP1A2 mRNA (Cui et al. 2009), which is possibly attributed to the fact that the expression of OATP1A2 is dependent on gestational ages (Loubière et al. 2010; Patel et al. 2003). In situ perfused rat placenta demonstrated that obstructive cholestasis impaired both [^{14}C] glycocholate placental transfer and maternal biliary secretion. UDCA (60 μg/day/100 g) moderately improved the latter but had a more marked beneficial effect on glycocholate placental transfer. Moreover, trophoblast apical plasma membranes demonstrated impairment in the ATP-dependent glycocholate transport across obtained from obstructive cholestasis placentas. In rats, it was found that obstructive cholestasis significantly induced expressions of placental Oatp1a1 and Oatp1a4 (but not Oatp1b2) mRNA, and that expressions of placental Oatp1a4 and Oatp1b2 were further enhanced by UDCA treatment. Histological examination revealed trophoblast atrophy and structural alterations, e.g., loss of apical membrane microvilli in obstructive cholestasis placentas, indicating that UDCA partially prevents deleterious effects of obstructive cholestasis on the rat placenta-maternal liver tandem excretory pathway, mainly by preserving trophoblast structure and function (Serrano et al. 2003).

OATPs, especially OATP1B3, mediate transport of unconjugated bilirubin in the placenta. Plasma membrane vesicles from human placenta tissue (Serrano et al. 2002) and cultured BeWo cells (Pascolo et al. 2001) demonstrated that OATPs mediated transport of unconjugated bilirubin across the placenta. Injection of mRNA of OATP1A2, OATP1B1, or OATP1B3 or RNA from human liver, human placenta, or mononucleated human trophoblast into *Xenopus laevis* oocytes also conferred on their ability to take up [^{3}H]$E_2$17βG and [^{3}H]-unconjugated bilirubin. Both unlabelled $E_2$17βG and unconjugated bilirubin cis-inhibited uptake of [^{3}H]$E_2$17βG and [^{3}H]-unconjugated bilirubin in all cases. The affinity and efficiency of [^{3}H]-unconjugated bilirubin uptake transport was OATP1B1 > OATP1B3. Kinetic parameters for [^{3}H]-unconjugated bilirubin uptake induced by RNA from mononucleated human trophoblast cells resembled most closely those of OATP1B3. Different from the liver, OATP1B1 is not or very lowly expressed in the placenta, indicating that OATP1B3 may play a major role in the carrier-mediated uptake of fetal unconjugated bilirubin by the placental trophoblast, whereas both OATP1B3

and OATP1B1 may substantially contribute to unconjugated bilirubin uptake by adult hepatocytes (Briz et al. 2003a). Animal experiments further demonstrated roles of OATPs in unconjugated bilirubin uptake. In single-pass perfusion of rat placenta, it was found that about 15% of perfused [^{3}H]-unconjugated bilirubin via umbilical artery was taken up by the placenta, which was inhibited by unconjugated bilirubin, bromosulfophthalein, cholic acid, or biliverdin (Briz et al. 2003b). Biliverdin perfused via umbilical artery was also taken by the placenta, where it was transformed into bilirubin and then transferred to the maternal blood. Injecting *Xenopus laevis* oocytes with mRNA from the placenta enhanced their ability to take up biliverdin. The expression of the Oatp isoforms in this system revealed that they have a varying degrees of ability to transport biliverdin (Oatp1a1 > Oatp1a4 > Oatp1b2). The abundance of their mRNA in rat trophoblast was Oatp1a1 > > Oatp1b2 > Oatp1a4, indicating that several members of the OATPs may contribute to the uptake of fetal biliverdin by the rat placenta (Briz et al. 2006).

OATP4A1 is highly expressed in the placenta. The triiodothyronine uptake by OATP4A1 was further characterized with an apparent K_m value of 0.9 μM. Compared with other OATPs (such as OATP1A2 and OATP1B3), OATP4A1 possesses rather high affinity to thyroid hormone and high expression in the placenta, indicating that OATP4A1 may play more important roles in the delivery of thyroid hormones from the maternal blood across the placenta to the fetus (Hagenbuch 2007). Thyroid hormone taken up by OATP4A1 on the microvillous membrane could then diffuse to the fetus via the facilitated transporters transporter TAT1 that is localized to the basal membrane of placental syncytiotrophoblast (Cleal et al. 2011).

OATPs also mediate transport of steroid sulfates or conjugates such as E3S and dehydroepiandrosterone sulfate (DHEAS) in the placenta. A report showed that OAT4, OATP2B1 and BCRP mediate transmembranal transfer of E3S and DHEAS in human placenta. Interestingly a significant correlation of BCRP and OATP2B1 but not OAT4 mRNA was found. In a MDCK cell model expressing both transport proteins simultaneously (OATP2B1 and BCRP in the basal and apical membrane, respectively), it was found that transport of both E3S and DHEAS significantly increased in basal-to-apical direction but not in the apical-to-basal direction. These data indicated that OATP2B1 localized in basal membrane and BCRP located in apical membrane of syncytiotrophoblasts in series work to accomplish transport of transepithelial transport of the steroid sulfate in human placenta (Grube et al. 2007). Although OAT4 and OATP2B mediate transport of steroid sulfates in placenta, the two transporters show different physiological roles in placental uptake of fetal-derived steroid sulfates. In HEK293 cell-expressed OAT4 or OATP2B1, uptake of E3S by OAT4- or OATP2B1-transfected cells was highly increased compared to the non-transfected cells. In contrast, DHEAS uptake was only highly increased in OAT4 but only weakly enhanced in OATP2B1 cells. The OAT4-mediated uptake of DHEAS and E3S was partly Na^+-dependent (about 50%), whereas uptake of DHEAS by OATP2B1 was Na^+-independent. Uptake kinetic parameters E3S by OAT4 and OATP2B1 were similar. The affinity of DHEAS toward OATP2B1 was about ten times lower than for OAT4 (Ugele et al. 2008).

11.6 Organic Anion Transporter 4 (OAT4/SLC22A11) and Blood-Placental Barriers

OAT4, coded by *SLC22A11* gene, is the only transporter specific for humans and expressed mainly in the placenta and kidney (Rizwan and Burckhardt 2007). OAT4-mediated transport is sodium-independent (Cha et al. 2000) and is coupled to the glutamate gradient (Lofthouse et al. 2015). The substrate specificity of OAT4 includes various steroid sulfates, such as E3S, beta-estradiol-3,17-disulfate, 17-beta-estradiol-3-sulfate, beta-estradiol-3-sulfate, and DHEAS (Zhou et al. 2003). Protein kinase C (PKC) activators phorbol 12-myristate 13-acetate and phorbol 12,13-dibutyrate acutely inhibited the transport activity (Zhou et al. 2006). In contrast, cAMP-dependent protein kinase A (PKA) activators 8-bromo-cAMP (a cAMP analog) and forskolin acutely stimulated the transport activity (Zhou et al. 2003; Tomi et al. 2014). OAT4-mediated transport of E3S was inhibited by several sulfate conjugates (such as p-nitrophenyl sulfate, alpha-naphthyl sulfate, beta-estradiol sulfate, and 4-methylumbelliferyl sulfate) but not glucuronide conjugates (Cha et al. 2000). Some therapeutic drugs such candesartan, candesartan cilexetil, losartan, losartan carboxyl, valsartan, pranlukast, and probenecid also inhibited OAT4-mediated transport of E3S (Yamashita et al. 2006). Pregnancy-specific hormone progesterone but not 7 beta-estradiol time- and concentration-dependently downregulated OAT4 transport activity, due to decreases in cell surface expression not expression of total protein. Activation of PKC by phorbol 12,13-dibutyrate also inhibited OAT4 activity via decreasing cell surface expression of OAT4 (Zhou et al. 2007), but progesterone-induced downregulation of OAT4 activity could not be prevented by treating OAT4-expressing cells with the PKC inhibitor staurosporine, indicating that progesterone regulates OAT4 activity by mechanisms independent of PKC pathway (Zhou et al. 2007).

OAT4 mRNA is highly and equally expressed in term placental tissue and in freshly isolated and cultured trophoblasts at term. OAT4 protein is abundantly expressed at the basal surface of the syncytiotrophoblast in terminal and intermediate villi. In the first trimester, OAT4 is also abundantly expressed at the basal surface of the syncytiotrophoblasts. Additionally, strong staining of the cytoplasm membrane and perinuclear region of cytotrophoblasts is detectable (Ugele et al. 2003). OAT4 localized at the basal surface of the syncytiotrophoblast mediates transport of steroid sulfates as OATP2B1 does (Ugele et al. 2003). Main functions of placental OAT4 are to transport steroid sulfates such as DHEAS, 16α-hydroxydehydroepiandrosterone sulfate (16α-OH DHEAS), and E3S. Both OAT4 and OATP2B1 mediate transports of DHEAS and E3S in the placenta. The affinity of E3S to OAT4 was similar to OATP2B1, but DHEAS is preferentially transported by OAT4. The affinity of OAT4 to DHEAS was about ten times higher than that of OATP2B1 (Ugele et al. 2008), indicating that the OAT4 and OATP2B1 show different physiological roles in placental uptake of fetal-derived steroid sulfates.

It is believed that estrogen biosynthesis in the placenta uses DHEAS, a precursor produced in large amount by the fetal adrenals. Moreover, accumulation of excess

DHEAS is associated with intrauterine growth retardation (Rabe et al. 1983). Therefore, OAT4 may play an important role in efficient uptake of DHEAS by the placenta for the production of estrogens and for the protection of fetus from the cytotoxicity of DHEAS. PKA is considered to be a key player in controlling syncytiotrophoblast formation. A report showed that forskolin increased expression of OAT4, leading to evoked uptakes of DHEAS and E3S, which were inhibited by a PKA inhibitor KT5720. Consistently, estradiol secretion from JEG-3 cells was occurred only in the presence of DHEAS in the medium, and the secretion was significantly suppressed by an OAT4 inhibitor bromosulfophthalein, suggesting the involvement of OAT4 in estradiol synthesis (Tomi et al. 2014). Similarly, PKA activator 12-O-tetradecanoylphorbol 13-acetate also increased the conversion of added DHEAS to estradiol in JEG-3 cells (Ritvos 1988), which is in line with increases in uptake of DHEAS due to induction of OAT4 (Tomi et al. 2014).

Estriol biosynthesis in human placenta requires the uptake of a fetal liver-derived estriol precursor, 16α-OH DHEAS, by placental syncytiotrophoblasts at their basal plasma membrane, which faces the fetal circulation. Stably transfected OAT4 HEK293 cells revealed a partly sodium-dependent transport for 16α-OH DHEAS with an apparent K_m of 23.1 μM and V_{max} of 485.0 pmol/mg protein/min, while stably transfected OATP2B1-HEK293 cells did not transport 16α-OH DHEAS at all (Schweigmann et al. 2014). Basal plasma membrane vesicles from human placental syncytiotrophoblasts demonstrated that characteristics of vesicles [^{3}H]16α-OH DHEAS uptake were in good agreement with those of OAT4-transfected COS-7 cells as well as forskolin-differentiated JEG-3 cells (Tomi et al. 2015). The JEG-3 cells are biochemically and morphologically differentiated into syncytiotrophoblast-like cells by forskolin. The forskolin-differentiated JEG-3 cells possess functional activity of OAT4 (Tomi et al. 2014). Little estriol secretion from JEG-3 cells was observed in the absence of estrogen precursors or in the presence of DHEAS, but secretion was greatly increased in the presence of 16α-OH DHEAS in the medium. Moreover, in the presence of 16α-OH DHEAS, co-incubation with bromosulfophthalein(50 μM) significantly decreased estriol secretion to 65% of that without bromosulfophthalein (Tomi et al. 2015), which was in line with the findings that bromosulfophthalein at 50 μM inhibited OAT4-mediated transport by more than 90% (Cha et al. 2000). These results demonstrate functional roles of OAT4 in the placental estriol synthesis (Schweigmann et al. 2014; Tomi et al. 2015). 16α-OH DHEAS concentration in umbilical artery at term is about 10 μM (Furuya et al. 1976), similar to the K_m of OAT4-mediated uptake of 16α-OH DHEAS (7.4 μM) in membrane vesicles of human placental syncytiotrophoblasts (Tomi et al. 2015), indicating that OAT4-mediated uptake is functionally able to supply the increasing demand for 16α-OH DHEAS in placental syncytiotrophoblasts toward the end of gestation.

OAT4 localized at basal plasma membrane vesicles of syncytiotrophoblast also mediates transport of some drugs and toxins in the placenta. For example, perfluorinated alkyl acids are widely used in industry and consumer products. Pregnant women are exposed to these toxins, and their presence in umbilical cord blood represents fetal exposure. Interestingly, these toxins are also substrates of

OAT4. Ex vivo dual recirculating human placental perfusion from eight placentas of healthy mothers demonstrated that ratios of fetal-to-maternal concentration of perfluorooctanesulfonate and perfluorooctanoate at the end of the perfusion were 0.26 ± 0.09 and 0.20 ± 0.04, respectively. The placental transfer indexes of the two compounds at 120 min were in negative correlation with the expression of OAT4 protein not BCRP. These results indicate that placental barrier limits transfer of perfluorinated alkyl acids, which is partly attributed to placental OAT4 (Kummu et al. 2015). In addition, OAT4 also mediates bidirectional transport of olmesartan in the placenta (Noguchi et al. 2015).

11.7 Other Drug Transporters and Blood-Placental Barriers

11.7.1 Organic Cation Transporters (OCT3/SLC22A3), Multidrug and Toxin Extrusion (MATEs/SLC47As), and Placental Barrier

In general, OCTs and MATEs, sharing substrate specificity, are localized in basal membrane and apical membrane of polarized cells, respectively. Functionally, they work in series to transport their substrates across cells from basal side to apical side. Several reports have demonstrated that among three OCTs (OCT1, OCT2 , and OCT3), only OCT3/Oct3 is abundantly expressed in the human, rat, and mouse placenta (Sata et al. 2005; Ahmadimoghaddam et al. 2012; Lee et al. 2013). In rats, Oct3 and Mate1 mRNA and protein were abundantly expressed in the placenta, which achieved significantly higher levels than those in the maternal kidney (Ahmadimoghaddam et al. 2012, 2013), but levels of human MATE1 mRNA were 1000-fold lower in the placenta than the kidney (Ceckova et al. 2016). mRNA of mouse Mate1 was mainly detected in fetal membranes (i.e., yolk sac and amniotic membranes), which was sevenfold higher than the placenta (Aleksunes et al. 2008). Immunohistochemical visualization revealed preferential localization of Oct3 on the basolateral side of the placenta, whereas Mate1 positivity was located in the labyrinth area predominantly on the apical side of the rat placenta (Ahmadimoghaddam et al. 2012; Lee et al. 2018). OCT3 positive staining was also observed in human fetal capillaries (Lee et al. 2018), but no Oct3 positive response was detected in rat fetal capillaries (Ahmadimoghaddam et al. 2013). In rat placenta, a strong positive Oct3 response was detected in the labyrinth area. Staining was predominantly visible in layers II and III of the syncytiotrophoblast but was not visible in layer I (Ahmadimoghaddam et al. 2012, 2013). Oct3 expression is only observed in the mouse syncytiotrophoblast II (Wu et al. 2016).

Expression of placental OCT3 and MATE1 is dependent on gestational ages with large species variability. The human OCT3 protein in first-trimester (weeks 6–12), second-trimester (13–25 weeks), and term placentas by LC-MS/MS was

measured to be 0.23 ± 0.033, 0.38 ± 0.072, and 0.36 ± 0.099 fmol/mg membrane protein (Lee et al. 2013), respectively, but mRNA of human OCT3 was reported to significantly decrease in term placentas compared with first trimester (Ahmadimoghaddam et al. 2013). In contrast with the moderate increase in human OCT3 protein during human pregnancy, Oct3 expression in the mouse placenta was highly dependent on gestational age. Compared with gestational day 10, placental mouse Oct3 mRNA increased by 37-fold and 46-fold at gestation day 15 and 19, leading to a 56-fold and 128-fold increase in Oct3 protein (Lee et al. 2013), respectively. Similar report demonstrated that mRNA of Oct3 in mouse placenta of gestation day 10 was 90% lower than that in gestation day 15 and 19 (Shuster et al. 2013). In rat placenta, Oct3 and Mate1 mRNA levels increased significantly toward the end of gestation, reaching values almost 9-fold and 17-fold higher in the term placenta compared with those on gestation day 12, but Mate2 levels remained unchanged throughout gestation. Oct3 and Mate1 protein expression in the placenta also showed an increasing trend toward the end of gestation, reaching 1.88- and 1.63-fold higher levels on gestation day compared with those on gestation day 12 (Ahmadimoghaddam et al. 2013).

The function of placental OCT3 has been demonstrated by model compound 1-methyl-4-phenylpyridinium (MPP^+). It was found that characteristics of MPP^+ uptake in human placental basal membrane vesicles were similar to that in HEK293 cells stably expressing OCT3 (Sata et al. 2005). Metformin is a substrate of OCT3 and MATE1. In dual perfusion of the rat placenta, the maternal or fetal side of the placenta was infused with various concentration of metformin (0.1 μM, 1 mM, or 10 mM) with a trace amount of [^{14}C]metformin. The results showed that transplacental clearance of metformin in maternal-to-fetal direction significantly increased with substrate concentration. In contrast, transplacental clearance of metformin in fetal-to-maternal direction significantly decreased with substrate concentration, confirming the involvement of a capacity-limited transport mechanism. At low metformin concentration, significant asymmetry between maternal-to-fetal direction and fetal-to-maternal direction in favor of the fetal-to-maternal direction was observed. The estimated fetal-to-maternal clearance at 0.1 μM metformin was 7.3 times higher than that in the opposite direction, but at high metformin concentration (10 mM), fetal-to-maternal and maternal-to-fetal clearances reached almost identical (Ahmadimoghaddam and Staud 2013). This asymmetry between maternal-to-fetal and fetal-to-maternal transport of MPP^+ in favor of fetal-to-maternal direction has further been confirmed in dual perfusion of the rat placenta using model compound MPP^+ (Ahmadimoghaddam et al. 2012). These results demonstrate that OCT3 localized at basal membrane of trophoblast uptakes organic cations into the trophoblast cell from fetal circulation and that MATE1 protein located at apical membrane effluxes these cations into the maternal circulation (Fig. 11.3). The common contribution of placental Oct3 and Mate1 to transfer of their substrates across the placenta was also demonstrated in vivo. A report showed that MPP^+ was infused intravenously into pregnant rats at gestation day 12, 15, 18, and 21; its concentrations at steady state in maternal blood sampling and fetus were measured. The results

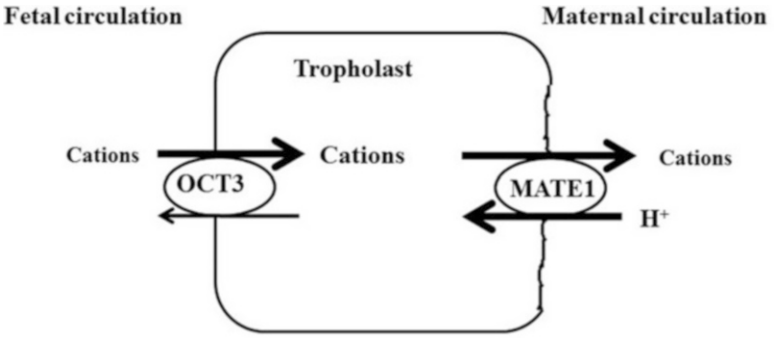

Fig. 11.3 Placental OCT3 and MATE1 work in series to transport cationic compounds across the placenta in the fetal-to-maternal direction. OCT3 and MATE1 are localized at basal membrane and apical membrane of trophoblast, respectively

showed that the amount of MPP^+ reaching the fetus on gestation day 15, 18, and 21 was approximately tenfold lower than on gestation day 12, which were consistent with low expression of placental Oct3 and Mate1 on gestation day 12, suggesting their protection of the fetus against maternal MPP^+ (Ahmadimoghaddam et al. 2013).

Roles of placental Oct3 were also confirmed using *Oct3*$^{-/-}$ pregnant mice. It was found that after oral dosing of [^{14}C]metformin at gestational day 19 of wild-type and *Oct3*$^{-/-}$pregnant mice, the systemic drug exposure ($AUC^{0-\infty}$) in maternal plasma was slightly reduced by ~16% in the *Oct3*$^{-/}$ pregnant mice, but fetal $AUC^{0-\infty}$ and fetal-to-maternal ratio were reduced by 47% and 44% in the *Oct3*$^{-/-}$ pregnant mice (Lee et al. 2018), respectively. Similarly, embryos of *Oct3*$^{-/-}$ pregnant mice showed a threefold reduction in MPP^+ compared with wild-type mice, although no differences in [^{3}H]MPP^+ accumulation were found in placentas and amniotic fluid of both groups (Zwart et al. 2001). These results demonstrate a significant role of Oct3 in facilitating cation fetal distribution and exposure during pregnancy. Moreover, OCT3 may be at least involved in transfer of serotonin from the maternal blood to the fetus (Kliman et al. 2018).

11.7.2 Organic Cation/Carnitine Transporters (OCTN2/SLC22A5) and Blood-Placental Barrier

OCTN2, a 63 kDa high-affinity carnitine uptake protein, is predominantly expressed localized in the apical surface of syncytiotrophoblast in human placenta (Grube et al., 2005; Lahjouji et al. 2004) and in the brush border membrane of BeWo cells (Huang et al. 2009). OCTN2-mediated L-carnitine uptake is sodium- and pH-dependent, showing high affinity for L-carnitine, which is inhibited by verapamil, valproate, tetraethylammonium, and pyrilamine and by structural analogs

of L-carnitine (including d-carnitine, acetyl-d,l-carnitine, and propionyl-, butyryl-, octanoyl-, isovaleryl-, and palmitoyl-L-carnitine) (Grube et al. 2005; Lahjouji et al. 2004). In BeWo cells, fluoroquinolones ciprofloxacin, gatifloxacin, ofloxacin, levofloxacin, and grepafloxacin were reported to inhibit L-carnitine transport, among which, grepafloxacin showed the strongest inhibition (Hirano et al. 2008). Genistein, progesterone, and alkaline phosphatase inhibitor levamisole inhibited OCTN2-mediated carnitine uptake, but this inhibition seemed to be not associated with their inhibition of phosphorylation (Rytting and Audus 2008).

Expression of OCTN2 also parallels cellular differentiation (Grube et al. 2005). It was reported that in BeWo cells, induction of syncytialization by forskolin significantly upregulated expression of OCTN2 protein, but did not alter V_{max} of carnitine uptake. Forskolin treatment downregulated expressions of PDZ domain-containing proteins PDZK1, NHERF1, and NHERF2 but not PDZK2 protein, indicating that the increase in uptake capacity by forskolin may be compensated by the decreased expression of PDZK1, NHERF1, or NHERF2 (Huang et al. 2009), which seems to explain why forskolin did not alter V_{max} of carnitine uptake, in despite of increases in expression of OCTN2 protein.

In early pregnancy, the placenta often develops under hypoxic status. Moreover, low placental oxygen concentrations in pregnancy are often linked to some complications such as preeclampsia and intrauterine growth restriction. It was found that OCTN2 and PPARα protein and mRNA levels were lower in syncytiotrophoblasts from preeclamptic human placentas than in those from normal placentas (Chang et al. 2011). In consistence, hypoxia decreased expression of OCTN2 and PPARα (mRNA and protein) in human placental explants, which was reversed by a PPARα agonist WY14643, indicating that hypoxia downregulates placental OCTN2 via PPARα-mediated pathway (Chang et al. 2011). It was consistent with alterations in expression of OCNT2 that hypoxia also significantly reduced OCTN2-mediated L-carnitine uptake (Chang et al. 2011; Rytting and Audus 2007). Moreover, hypoxia impaired induction of syncytialization by forskolin and decreased the forskolin-induced increases in OCTN2 expression (Chang et al. 2011).

Physiological function of OCTN2 is to mainly mediate L-carnitine uptake. L-Carnitine plays an important role in lipid metabolism by facilitating the transport of long-chain fatty acids across the mitochondrial inner membrane followed by fatty acid beta-oxidation. Placental trophoblast cells oxidize long-chain fatty acids for energy production, indicating that OCTN2 seems to be involved in fatty acid beta-oxidation in the placenta. A report showed that the fetal and placental weights were similar among the three genotypes ($Octn2^{+/+}$, $Octn2^{+/-}$, and $Octn2^{-/-}$) and the levels of carnitine were markedly reduced (<20%) in fetuses and placentas of $Octn2^{-/-}$ mice compared with wild-type controls. Catalytic activities of beta-oxidation enzymes were present at comparable levels in placentas of $Octn2^{+/+}$ and $Octn2^{-/-}$ mouse, but activity of short-chain 3-hydroxyacyl-CoA dehydrogenase was significantly higher in $Octn2^{-/-}$ placentas than in $Octn2^{+/+}$ placentas. These data indicate that placental OCTN2 is obligatory for accumulation of L-carnitine in the placenta and fetus and that reduced L-carnitine levels upregulate the expression of short-chain 3-hydroxyacyl-CoA dehydrogenase in the placenta (Shekhawat et al. 2004). Another report also showed that L-carnitine deficiency due to a defect in the

carnitine transporter Octn2 in a mouse model leads to embryonic lethality. Placental levels of L-carnitine are reduced to <10% of normal, and deficiency of L-carnitine is associated with markedly reduced expression of several growth factors and transforming growth factor β-genes, demonstrating that the reduced L-carnitine levels in the placenta are linked to embryonic lethality (Shekhawat et al. 2018).

Sulpiride is a typical antipsychotic drug for the treatment of schizophrenia, depression, and other psychological disorders. Sulpiride was a substrate of OCT3, P-GP, and BCRP. The accumulation of sulpiride in BeWo cells was obviously affected by inhibitors of OCTN2, P-GP, and BCRP. Inhibitors of OCT3, OCTN1, and OCTN2 obviously decrease the accumulation of sulpiride in primary human trophoblast cells. These results indicate that OCTN1 and OCTN2 likely contribute to the sulpiride uptake from maternal circulation to trophoblast cells, while P-GP and BCRP mediate the efflux from trophoblast cells to maternal circulation, and OCT3 probably is involved in the bidirectional transport of sulpiride between the placenta and fetal blood (Bai et al. 2017). OCNT2 is also involved in entecavir transport in the placenta (Ma et al. 2017).

11.7.3 Equilibrative Nucleoside Transporters (ENTs/SLC29As) and Blood-Placental Barrier

The identified nucleobase transporters in both rat and human placenta include equilibrative nucleoside transporters (ENT1/SLC29A1 and ENT2/SLC29A2) and concentrative nucleoside transporters (CNTs/SLC28As) (Nishimura and Naito 2005; Leazer and Klaassen 2003; Jiraskova et al. 2018). CNTs mediate a unidirectional influx coupled with a Na^+ gradient, and ENTs mediate a bidirectional flux across the plasma membrane depending on the substrate concentration gradient. The role of placental ENTs has been widely investigated. ENTs are typically divided into two classes ENT1 and ENT2 based on their sensitivity or resistance, respectively, to inhibition by nitrobenzylthioinosine: ENT1 is sensitive to nitrobenzylthioinosine with K_I values of 0.1–10 nM. ENT2 is insensitive to nitrobenzylthioinosine, whose K_I value is larger than 1 μM (Yao et al. 1997; Griffiths et al. 1997). ENT2 is much less sensitive to inhibition by dipyridamole, dilazep, and draflazine (Griffiths et al. 1997). The amino acid sequence of human ENT2 is 46% identical to that of human ENT1 (Griffiths et al. 1997). Rat Ent1 was 78% identical to human ENT1 in amino acid, and rat Ent2 was 49–50% identical to rat Ent1/human ENT1 (Yao et al. 1997). ENT1 is localized in the apical membrane of human syncytiotrophoblast (Barros et al. 1991; Barros et al. 1995; Govindarajan et al. 2007), the lumenal surfaces of fetal capillaries, small placental vessels, and umbilical vein (Barros et al. 1995). ENT2 is found in the outer trophoblast layer of human term placenta (Govindarajan et al. 2007). mRNA of Ent1 and Ent2 was detected in conditionally rat immortalized syncytiotrophoblast cell lines TR-TBT 18d-1 and TR-TBT 18d-2 (derived from syncytiotrophoblasts I and II in rat,

respectively) (Lee et al. 2011). First- and third-trimester placentas demonstrated that human ENT1 is the dominant isoform in human placenta. However, a report demonstrated no significant difference in expression of ENT1 and ENT2 over the course of placental development, which was due to considerable interindividual variability. Similarly, the expression profile of Ent2 mRNA in rat placenta during pregnancy was almost constant from 13.5 to 21.5 days of gestation (Takagi et al. 2017). Significant activity of ENT1 and CNT2 was found in BeWo cells, but the villous fragments and microvillous plasma membrane vesicles (apical membrane of a syncytiotrophoblast) only showed ENT1 activity (Cerveny et al. 2018).

Some diseases affect expression of placental ENTs. Human placental microvascular endothelium showed that in normal pregnancies, ENT1-mediated adenosine transport was higher than ENT2-mediated adenosine and preeclampsia significantly decreased ENT1-mediated adenosine transport with decreases in V_{max} by 70% of normal pregnancies. In contrast, preeclampsia significantly increased V_{max} of ENT2-mediated transport to 2-fold of normal pregnancies, without affecting its apparent K_m. The net effect was that total transports (human ENT1 + human ENT2) increased twofold. Further study demonstrated that preeclampsia decreased expression of ENT1 (by 78% for protein and by 63% for mRNA) and increased expressions of ENT2 (5.2-fold for protein and 4.8-fold for mRNA) compared with normal pregnancies (Escudero et al. 2008). It was also found that extracellular adenosine concentration was significantly higher (4.1-fold) in the culture medium of human placental microvascular endothelium from preeclampsia compared with normal pregnancies, which may be due to either reduces in adenosine uptake or increases in release of adenosine from the human placental microvascular endothelium of preeclampsia. Clinical reports demonstrated that plasma adenosine concentration in preeclampsia was significantly higher than in normal pregnancy (Takeuchi et al. 2001) and that the plasma adenosine levels were positively correlated to severity of preeclampsia (Yoneyama et al. 2002), all of which also contribute to the increased adenosine level detected in the umbilical vein blood in preeclampsia (Yoneyama et al. 1996; Yoneyama et al. 2002). The increased plasma concentrations of adenosine in preeclampsia may serve to counteract further progression of the complication (Yoneyama et al. 2002).

Gestational diabetes mellitus (GDM) is often linked to with micro- and macrovascular endothelial dysfunction with serious consequences for the growing fetus, which is partly attributed to nitric oxide (NO)-reduced adenosine uptake in placental endothelium (Guzmán-Gutiérrez et al. 2014; Vásquez et al. 2004). Human placenta microvascular endothelial cells demonstrated that gestational diabetes mellitus significantly reduced expression of ENT1 and ENT2 (protein and mRNA) by 62%–65% although a report showed that neither [^{3}H] nitrobenzylthioinosine binding, a marker of the facilitative-diffusion nucleoside transporter in the human placenta, nor adenosine metabolism exhibited a significant difference in either the brush border or the basal membrane vesicles of placentas between the normal and diabetic group (Osses et al. 1995). In line with reduces in expression of ENT1 and ENT2, overall adenosine transport was significantly reduced in human placenta

microvascular endothelial cells from GDM compared with normal pregnancies. These results indicate that GDM is a syndrome associated with reduced overall adenosine transport due to reduced expression and activity of human ENT1 and human ENT2 in the placenta (Salomón et al. 2012). These results may also explain the fact that diabetes impaired responses to adenosine in both chorionic arteries and veins, altering a systemic adenosine signaling in the fetoplacental circulation (Razak et al. 2018).

The placenta requires nucleosides as nutrients for fetal growth, so it is important to examine potential interactions between placental transports of nucleosides and drugs to ensure the safety of pharmacotherapy during pregnancy. ENT1 and ENT2 accept various drugs that are structurally related to nucleosides as substrates and/or inhibitors, and so there is potential for DDIs, especially in antiretroviral treatment because three or more antiretroviral drugs are often administered at the same time. [^{3}H]uridine or [^{3}H]adenosine uptake from the apical side of TR-TBT 18d-1 is mediated by Ent1 and Ent2. It was reported that their uptakes were significantly reduced by high concentrations of several nucleoside drugs, including cytarabine, vidarabine, zidovudine, mizoribine, caffeine, and amitriptyline, but their inhibitory effects were small within the therapeutic concentration ranges (Chishu et al. 2008). Fluorouracil is used for treatment of breast cancer even in pregnant women. TR-TBT 18d-1 cells demonstrated that Ent2 mediates fluorouracil, didanosine, and 6-mercaptopurine uptake (Takagi et al. 2017; Lee et al. 2011; Sato et al. 2009), indicating that ENT2 may contribute to their uptake at the blood-placental barrier. BeWo cells and vesicles of apical membrane of syncytiotrophoblast also demonstrated that ENT1 plays the dominant role in abacavir uptake into placental tissues. Dually perfused rat term placentas further demonstrated that Ent1 contributes significantly to overall [^{3}H]-abacavir placental transport (Cerveny et al. 2018). Ribavirin is used for the treatment of hepatitis C virus infection. The ribavirin transports into the liver is mediated by ENT1. A report showed that at 15 min after intravenous [^{3}H]-ribavirin (3 mg/kg) administration to *Ent1*$^{-/-}$, *Ent1*$^{+/-}$, and *Ent*1$^{+/+}$ pregnant mice, compared with the *Ent1*$^{+/+}$ mice, the fetal distribution of ribavirin at 15 min after administration was significantly reduced in *Ent1*$^{-/-}$ fetuses and placenta. The ratios of tissues-to-maternal concentrations were *Ent1*$^{-/-}$mice< E*nt1*$^{+/-}$ mice <*Ent1*$^{+/+}$ mice (Endres et al. 2016), demonstrating roles of ENT1 in placental and fetus distribution of ribavirin. The cord plasma concentrations of didanosine and zalcitabine are approximately 50% of their maternal concentrations. ENT1 and ENT2 also mediate transport of didanosine and zalcitabine (Tomi et al. 2011), indicating that mother-to-fetus transfer of the two compounds may be modulated by ENT2 and ENT1.

It should be noted that transport of most drugs in the placenta is mediated by many transporters. For example, entecavir is substrate of ENT1, ENT2, CNT2, CNT3, OCT3, OCTN2, MRP2, P-GP, and BCRP, demonstrating that net transport of entecavir across placenta barrier is attributed to common effects of these transporters (Ma et al. 2017). CNT2, CNT3, ENT1, ENT2, and OCTN2 contributed to the entecavir uptake from maternal circulation to trophoblast cells, whereas OCT3

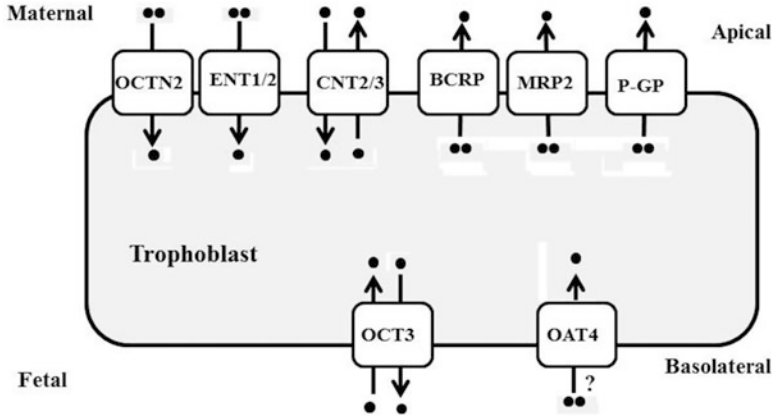

Fig. 11.4 SLC transporters (such as OCTN2, ENT1, ENT2, CNT2, CNT3, OCT3, and OAT4) and ABC transporters (such as BCRP, MRP2, and P-GP) work in series to mediate transport of entecavir across blood-placental barrier

mediates transport of entecavir efflux from trophoblast cells to fetal circulation or from fetal circulation to trophoblast cells. BCRP, MRP2, and P-GP may mediate efflux of entecavir from trophoblast cells to maternal circulation (Fig. 11.4). A recent report showed that expressions of both P-GP and BCRP were increased in the placenta of women infected with hepatitis C virus, in turn, impacting fetal exposure to P-GP or BCRP substrates (including entecavir) in pregnancies infected with infections (Pfeifer et al. 2018).

Moreover, there exist large interspecies differences in expression of drug transporters and characteristics of the placenta. In rodents, the endoplacental yolk sac is the major site of Mrp1 expression, but this has no structural equivalent in humans after the first trimester (Jauniaux et al. 2003). The MRP2 protein was immunolocalized to the apical syncytiotrophoblast membrane (Evseenko et al. 2006) in human placental villi but was undetectable when rat placentas were examined in the same way. Such discrepancies must be stressed when extrapolating data on fetal-maternal transport of certain solutes from the rodent model to humans. Results from various in vitro models (including cell line and human placental perfusion) are often different from findings in human, which raises the question of how to best study transport to the fetus. Therefore to fully understand transplacental transfer mechanisms of drugs via placental barrier, transporters should enable us to better control the transplacental transfer of drugs and thereby to minimize the potential fetal risk from drug exposure.

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Chapter 12
ABC Transporter-Mediated Multidrug-Resistant Cancer

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Abstract ATP-binding cassette (ABC) transporters are involved in active pumping of many diverse substrates through the cellular membrane. The transport mediated by these proteins modulates the pharmacokinetics of many drugs and xenobiotics. These transporters are involved in the pathogenesis of several human diseases. The overexpression of certain transporters by cancer cells has been identified as a key factor in the development of resistance to chemotherapeutic agents. In this chapter, the localization of ABC transporters in the human body, their physiological roles, and their roles in the development of multidrug resistance (MDR) are reviewed. Specifically, P-glycoprotein (P-GP), multidrug resistance-associated proteins (MRPs), and breast cancer resistance protein (BCRP/ABCG2) are described in

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more detail. The potential of ABC transporters as therapeutic targets to overcome MDR and strategies for this purpose are discussed as well as various explanations for the lack of efficacy of ABC drug transporter inhibitors to increase the efficiency of chemotherapy.

Keywords ABC transporters · Multidrug resistance · P-glycoprotein · Multidrug resistance-associated protein · Breast cancer resistance protein

12.1 Introduction

ATP-binding cassette (ABC) transporters are a large group of highly conserved transmembrane proteins that mediate the active transport of many structurally and functionally diverse substrates through the bilayer of the cell membrane. ABC transporters usually consist of two nucleotide-binding domains (NBDs), also known as ATP-binding domains, and two transmembrane domains (TMDs) bundled with 6–11 membrane-spanning α-helixes that determine substrate specificity (Dean and Allikmets 1995). The NBDs, located in the cytoplasm, include the conserved Walker A and B motifs, signature C motif, and A, Q, D, and H loops (Wlcek and Stieger 2014). The NBDs dimerize in a head-to-tail orientation, and ATP binds at the interphase. The functional ATP site is formed by the Walker A and B motifs of NBD1 and the signature region of NBD2. These transporters are found in all living organisms, from prokaryotes to eukaryotes. They transport ions, sugars, vitamins, nucleosides, amino acids, peptides, lipids, as well as other organic and inorganic molecules (Dean and Allikmets 1995). The ABC superfamily has been categorized into seven subfamilies (A to G), based on their structural and sequence similarities (Vasiliou et al. 2009).

Currently, there are 48 known ABC genes in the human genome, and mutations in the expression levels of over 20 of these transporters are involved in the pathogenesis of various human diseases, including cystic fibrosis, gout, Dubin-Johnson syndrome, adrenoleukodystrophy, central nervous system disorders, cancer, and other diseases (Wilkens 2015). For example, ABCA1 may play a role in the pathogenesis of Alzheimer's disease, and ABCA4 is linked to Stargardt disease (age-related macular degeneration) (Peca et al. 2015; Li et al. 2015). Specifically, mutations in ABCA1 have been shown to be involved in the development of Tangier disease, a rare illness associated with a significant deficiency in high-density lipoprotein (HDL) (Wang and Smith 2014). The ABCC subfamily is made up of 12 transporters (ABCC1 (MRP1), ABCC2 (MRP2), ABCC3 (MRP3), ABCC4 (MRP4), ABCC5 (MRP5), ABCC6 (MRP6), ABCC7 (CFTR), ABCC8 (SUR1), ABCC9 (SUR2A, SUR2B), ABCC10 (MRP7), ABCC11 (MRP8), and ABCC12 (MRP9)) (Linton and Holland 2011). ABCC7 (also known as the cystic fibrosis transmembrane conductance regulator or CFTR) is an ATP-dependent chloride ion channel found in lung cells and/or tissue. It is well established that several point mutations in *ABCC7* affect the phosphorylation sites of CFTR, rendering the channel defective (Bose et al. 2015; Pollock et al. 2015). Mutations in the ABCC2 transporter are associated with Dubin-Johnson syndrome (Slachtova et al. 2016). The D

Table 12.1 Major MDR-linked ABC drug transporters, their known drug substrates, and physiological locations

Transporter	Substrates	Where expressed
P-GP (ABCB1)	Anthracyclines, taxanes, vinca alkaloids, neutral and cationic organic compounds, digoxin, tyrosine kinase inhibitors	Intestine, kidney, adrenal, liver, testes, placenta, blood-brain barrier, hematopoietic and stem cells
BCRP (ABCG2)	Anthracyclines, alkylating agents, camptothecin-derived analogs, pheophorbide A, uric acid, flavopiridol, tyrosine kinase inhibitors	Intestine, kidney, placenta, liver, blood-brain barrier, breast, stem cells
MRP1 (ABCC1)	Anthracyclines, vinca alkaloids, epipodophyllotoxins, LTC_4, methotrexate, GS-X and other conjugates, organic anions	Widespread (basolateral surface of epithelial cells)

subfamily (ABCD) of transporters has been reported to play a crucial role in X-linked adrenomyeloneuropathy or adrenoleukodystrophy, as well as liver disease (Theodoulou and Kerr 2015; Stefkova et al. 2004). Table 12.1 provides an overview of the physiological locations of major ABC drug transporters and their known drug substrates.

The primary physiological function of some of the ABC transporters, which are expressed on the plasma membrane, is to mediate the efflux of toxic endogenous substances and xenobiotics from various cells and organs (Linton 2007). These transporters are also involved in the absorption and disposition of clinically administered drugs (Linton 2007). For example, their presence in tissues and organs can significantly decrease the levels of certain drugs, thereby diminishing or even abolishing their efficacy (Szakacs et al. 2008). The overexpression of certain ABC transporters in various cancers has been shown to produce multidrug resistance (MDR) and loss of efficacy of chemotherapy (Choi 2005). It is well established that members of the ABCB, ABCC, and ABCG subfamilies are major players in the development of MDR, a phenomenon characterized as resistance to anticancer drugs with different chemical structures and mechanisms of action (Chang 2003). The MDR-linked ABC transporters, namely, ABCB1/P-glycoprotein (P-GP), ABCG2/breast cancer resistance protein (BCRP), and ABCC1/multidrug resistance-associated protein 1 (MRP1), are asymmetrically distributed when they are present in the same cell. For example, in intestinal cells and kidney cells, P-GP and BCRP are located at the apical surfaces (brush borders), whereas MRP1 is located at the basolateral surface (Daood et al. 2008). These transporters also have varying tissue localization profiles. P-GP is localized in the intestine, liver, kidney, placenta, and blood-brain barrier, while ABCG2 is localized in the placenta, blood-brain barrier, small intestine, and stem cells (Mao and Unadkat 2015). MRP1 is expressed more ubiquitously in epithelial cells throughout the body and is expressed at the basolateral membrane in polarized cells (Daood et al. 2008).

12.2 Physiological Roles of ABC Transporters

As mentioned above, the primary physiological role of ABC transporters is to efflux or remove both endogenous and xenobiotic molecules from cells and organs. These transporters are present on membranes lining tissues such as the liver, lungs, gastrointestinal tract, the blood-brain barrier (BBB), the blood-retina barrier (BRB) and the placenta (Dean et al. 2001).

The active transport mediated by ABC transporters utilizes the hydrolysis of ATP to move substrates against the concentration gradient. Although there is no consensus concerning the stoichiometry of ATP hydrolysis to drug transport, it appears that two ATP molecules are needed to transport a single molecule across the membrane. The transport process is not limited to moving substances into and out of cells but also involves the movement of molecules intracellularly from the cytoplasm to organelles (Stefkova et al. 2004).

Many ABC transporters were found to be expressed by different types of human adult normal and malignant liver cells, where they aid in hepatic metabolism of xenobiotics. ABCB1, ABCB4, and ABCB11 from the B subfamily; ABCC2, ABCC3, and ABCC4 from the C subfamily; and ABCG2 from the G subfamily are localized in the human adult liver (Stindt 2011; Nicolaou et al. 2012). For example, P-GP transporters on hepatocyte and cholangiocyte liver cells are responsible for the transport of bulky neutral and cationic compounds into the bile (Chan et al. 2004). P-GP is upregulated in hepatic cell carcinomas (HCCs) due to the pathogenesis of the disease and in response to chemotherapy (Wlcek and Stieger 2014). In HCC, the expression of the ABCC1 (MRP1) transporter was found to be increased by 50% (Vander Borght et al. 2008). Finally, the expression level of ABC transporters in liver tumors is variable, making it difficult to predict the response of a particular patient to chemotherapy (Nies et al. 2001).

The kidneys play a vital role in the excretion of numerous xenobiotics (Anders 1980). ABC transporters mediate drug efflux and active secretion in the proximal tubules (Masereeuw and Russel 2012). The expression level of these transporters can be altered in tubular dysfunction and other disease conditions, resulting in a significant decrease in the efflux of drugs and toxic compounds, thereby increasing the likelihood of toxicity, depending on the drug (Wu et al. 2011b). Members of the ABC B, C, and G subfamilies are highly expressed in the kidneys (Masereeuw and Russel 2010). These transporters are localized in the proximal tubules and primarily mediate active efflux of certain molecules (Russel et al. 2008). The P-GP transporter is mainly localized at the apical membrane of the proximal tubules, where it mediates the efflux of many uncharged and cationic compounds (Hauser et al. 2005). The expression level of P-GP can be altered by certain drugs and disease conditions. For example, treatment with the immunosuppressant cyclosporine A increases the expression of P-GP (Hauser et al. 2005).

MRP transporters belonging to the ABCC superfamily play an important role in the excretory function of the kidney (van de Water et al. 2005). These transporters mainly mediate the excretion of organic anions (Chen and Tiwari 2011; van de Water et al. 2005). MRP2 is expressed primarily in proximal tubules, and its

deficiency is linked to the development of the Dubin-Johnson syndrome, which results in significant alteration of the secretion of several substrates, including glutathione (Masereeuw and Russel 2012). MRP4 is also highly localized primarily in the proximal tubules and effluxes a wide range of drugs, such as chemotherapeutic agents (methotrexate, 6-thioguanine, 6-mercaptopurine, topotecan), diuretics (furosemide, hydrochlorothiazide), antivirals (adefovir, tenofovir), antibiotics (cephalosporins), and antihypertensives, among others. Increased susceptibility to renal toxicity and nephrotoxicity has been reported in patients with deficiencies in MRP2 and MRP4 (van Aubel et al. 2002). MRP1, MRP3, MRP5, and MRP6 are expressed in other regions of the kidney, including the loop of Henle, the proximal convoluted tubules, and the distal and collecting ducts, and are involved in renal excretion of organic anions (van de Water et al. 2005).

Finally, another subfamily of ABC transporters that is involved in drug efflux is the G subfamily, with ABCG2 being the most recognized member, which is localized in the proximal tubules (Huls et al. 2008). ABCG2 actively effluxes organic anions and uncharged molecules (Huls et al. 2008). Certain polymorphisms such as Q141K in ABCG2 have been shown to significantly decrease the secretion of uric acid, producing hyperuricemia, resulting in gout (Woodward et al. 2009).

ABC transporters are present in the placenta, where they play a vital role in the transplacental exchange of nutrients between the mother and the fetus (Joshi et al. 2016). The placenta serves as a partition between maternal and fetal blood circulation (Tetro et al. 2018). It restricts the entry of certain toxic substances to the fetus but also secretes physiological compounds such as hormones and prostaglandins (Joshi et al. 2016; Moe 1995; Smith et al. 1992). The human placenta consists of polarized cytotrophoblasts and syncytiotrophoblasts, and ABCA, ABCB, ABCC, and ABCG transporters are located at the apical surface, where they efflux waste products and xenobiotics from the fetal blood into the maternal circulation for removal (Schmid et al. 2003). It was reported that physiological molecules, such as cholesterol and phospholipids, were transported from the maternal circulation to the fetal compartment by ABCA1 in the trophoblast-derived choriocarcinoma cell line (BeWo cells) which is used as an in vitro model for the placenta (Woollett 2011). Alterations in the expression of ABCA1 are linked to abnormalities in placentation, and mutations in ABCA1 produce HDL deficiency (Lawn et al. 1999; Marcil et al. 1999; Brooks-Wilson et al. 1999; Joshi et al. 2016). Guay et al. reported that the in utero environment modifies the DNA methylation profile of ABCA1, altering the lipid composition and increasing the risk of cardiovascular diseases in the fetus (Guay et al. 2012). These findings illustrate the need for epigenetic factors to be taken into consideration when determining the factors involved in ABC transporter-mediated diseases.

The central nervous system, in particular, the blood-brain barrier (BBB), acts as an effective shield to prevent toxic substances from entering the brain (Serlin et al. 2015). The brain is separated from the blood by the BBB and from the cerebrospinal fluid (CSF) by the choroid plexus (CP). The main function of the CP is to produce CSF. The CP also plays an adjunct role in removing organic anions, drugs, and drug metabolites from the CSF. The ABC transporter proteins expressed at the BBB

efflux various drugs and endogenous molecules. ABC transporters, such as P-GP and MRP1, are expressed in the human CP (Stieger and Gao 2015). While messenger RNA (mRNA) expression in humans has been studied intensively, little is known concerning the subcellular expression of transporters in specific cells in the CNS or regarding the barriers shielding the CNS from the systemic circulation. The role of transporters in the BBB and CP has been elucidated using knockout animal models (Stieger and Gao 2015). The use of such models can provide useful information about the physiological function of the transporters. The P-GP and ABCG2 transporters in brain capillaries contribute significantly to the barrier function of the BBB. For example, ivermectin produces neurotoxicity in Abcb1a-deficient mice, whereas ivermectin is nontoxic in wild-type mice. This is due to more than a 100-fold accumulation of ivermectin in abcb1-deficient, compared to wild-type, mice. Knocking out the *Mdr1a* gene in mice did not produce negative physiological effects (Stieger and Gao 2015; Schinkel et al. 1994, 1997). However, the knockout mice retained higher levels of certain drug substrates compared to controls. Using Abcg2 knockout mice, it was also shown that a single drug can be transported by multiple transporters (Vlaming et al. 2009). P-GP and ABCG2 were found to transport topotecan in opposite directions in the BBB and CP, as topotecan levels were increased in the brain parenchyma but decreased in the CSF (Shen et al. 2009a). Stieger and Guo highlighted the need for specific antibodies to define the subcellular expression of ABC transporters at brain barriers. The development of in vivo imaging probes and detection tools is also needed for investigating the level of ABC transporters present at the barriers in the CNS.

Additionally, studies have shown significant alterations in the expression levels of ABC transporters at the BBB as a result of neurological diseases such as Parkinson's disease, stroke, epilepsy, and Alzheimer's disease (Miller 2015). For example, the expression of P-GP is increased in animal models of epilepsy and stroke (Elali and Hermann 2012; Potschka and Luna-Munguia 2014). However, the mechanisms involved in producing these changes are complex and involve several signaling pathways (Miller 2015).

The presence of ABC transporters at the blood-brain barrier protects the brain from the accumulation of toxic endogenous and exogenous molecules. However, the transporters can significantly lower the concentration of therapeutic drugs in the brain, which can decrease or even abolish their clinical efficacy (Miller 2015). Furthermore, drugs that have sufficient lipophilicity to passively cross the membrane and reach the CNS may also be effluxed by ABC transporters (Löscher and Potschka 2005). For example, anticancer drugs such as doxorubicin, vinblastine, vincristine, and etoposide are effluxed by ABC transporters and have lower brain concentrations than expected. Other drugs, such as immunosuppressive agents (cyclosporine A), corticoids, analgesics (morphine), antibiotics (erythromycin, tetracyclines, and fluoroquinolones), antiepileptic (phenytoin), cardiac glycosides (digoxin), and

antiemetics (domperidone, ondansetron), among others, are substrates for various ABC transporters (Löscher and Potschka 2005). There are studies indicating that expression of specific ABC transporters in the BBB can result in the development of resistance to anticonvulsant drugs (Loscher et al. 2011).

ABC transporters are also located in the retina (Molday et al. 2009). The retina has two anatomical barriers: (1) the inner blood-retina barrier (BRB), formed by the retinal capillary endothelial cells, and (2) the outer BRB, formed by the retinal pigmented epithelial cells (Campbell and Humphries 2012). These barriers prevent uncontrolled entry of blood constituents into the eye (Díaz-Coránguez et al. 2017). The human BRB contains at least one member of the ABCB and ABCC families (Stieger and Gao 2015). The exact role of the transporters in drug permeation through the BRB is still largely unknown.

Human skin is the largest organ in the body (6% of body weight), and one of its functions is to be a physical as well as a chemical barrier for numerous environmental molecules (Takechi et al. 2018). Little is known about the expression levels, distribution, and function of ABC transporters in the skin (Takenaka et al. 2013; Osman-Ponchet et al. 2014). It has been reported that ABC transporters such as ABCA7 are highly expressed in human keratinocytes (Kielar et al. 2003). Previous studies suggested that the role of ABC transporters in the skin may include immune function, active efflux, and the progression and metastasis of melanoma (Osman-Ponchet et al. 2014).

P-GP is present in skin keratinocytes of neonatal and adult mice, as well as in extracts of human skin organotypic raft cultures (Li et al. 2010; Ito et al. 2008). As mentioned above, the exact function of these transporters is not completely understood. However, we have reported that P-GP has an absorptive function in skin keratinocytes where it effluxes various xenobiotics from the skin surface to the dermis and subcutis (Li et al. 2010). Furthermore, our results indicated that the anticancer effects of Ing3A, a skin cancer chemotherapeutic agent, are significantly enhanced by the absorptive activity of cutaneous P-GP transporters in the skin (Li et al. 2010). Another study reported the expression of several members of the A, B, and C subfamilies, especially ABCA5, ABCB3, ABCC4, and ABCC5 (Takenaka et al. 2013). Other transporters are also expressed in the skin but to a lesser and more variable extent (Takenaka et al. 2013). The interindividual variability in the expression level of ABC transporters appears to result in significant differences in drug transport and responses (Takenaka et al. 2013). Finally, the expression level of these transporters was affected by inflammatory mediators present in several dermatological diseases (Osman-Ponchet et al. 2014). For example, the gene expression levels of ABCC1 were significantly increased by melanomas and skin psoriasis (Smith et al. 2003; Depeille et al. 2005). The expression level can be also altered by certain drug treatments, such as the administration of dexamethasone and rifampicin (Osman-Ponchet et al. 2014).

12.3 Multidrug Resistance and ABC Transporters

12.3.1 Discovery of Transporters and Roles in Resistance

ABC transporter-mediated drug resistance was first discovered in 1973, when Dano et al. reported that Ehrlich ascites tumor cells became resistant to daunorubicin due to the overexpression of an ABC transporter. These cells were not only resistant to daunorubicin, but they were also cross-resistant to other chemotherapeutic compounds, such as vinca alkaloids and other anthracyclines (Dano 1973). Three years later, a 170-kDa cell membrane protein was identified that was expressed at the surface of the drug-resistant cells (Juliano and Ling 1976), and its expression was positively correlated with the magnitude of drug resistance in several cell lines. This protein was named P-glycoprotein (permeability-associated glycoprotein), and the gene encoding this protein was cloned in 1986 and named MDR1 (Ueda et al. 1987). Several research groups later demonstrated that the expression of P-GP was positively correlated with multidrug resistance in multiple cell lines (Lehne 2000). However, the functional correlation of P-GP expression with the emergence of drug resistance in the clinic was not established. Also, no detectable expression of P-GP was observed in some drug-resistant cancers, prompting the search for other complex mechanisms that could produce the MDR phenotype in the absence of P-GP.

In 1992, the research group of Cole and Deeley discovered another ABC transporter, the multidrug resistance protein (MRP1), which conferred resistance to antimetabolite or alkylating anticancer drugs that were not substrates of P-GP (Cole et al. 1992). The MRP1 transporter shares similar structural characteristics with P-GP, except for the presence of an extra membrane-spanning domain, TMD0, at the N-terminus of the transporter. However, MRP1 was shown to transport certain anticancer drugs conjugated with glutathione. A major difference between P-GP and MRP1 substrates is that while P-GP substrates are neutral or slightly basic organic compounds, MRP primarily transports drugs conjugated with sulfate, glucuronate, or glutathione, including methotrexate, arsenite, or cisplatin (Borst et al. 2000). This organic anion transport activity of MRP1 was attributed to the presence of an extra transmembrane domain in MRP1 (Borst et al. 2000). As demonstrated for P-GP, the expression levels of MRP1 were correlated with the presence of the drug resistance phenotype in in vitro cultured cells (Cao et al. 2017). Several other members of the MRP subfamily have since been identified (Kruh et al. 2001). MRP2, with substrate specificity overlapping that of MRP1, mediates bilirubin glucuronide transport, and its functional inactivation results in the Dubin-Johnson syndrome. This transporter, similar to P-GP, is localized to the apical surface of polarized epithelial cells. MRP3 is expressed at high levels in the liver and catalyzes the efflux of organic anions including etoposide and vincristine from the liver to the blood in the presence of biliary obstruction (Zeng et al. 1999). MRP4 and MRP5 were found to transport antiretroviral nucleoside analogs and thiopurine-based anticancer drugs. MRP4 has been reported to produce resistance to nucleoside analogs (Kruh et al. 2001). Furthermore, the expression of both MRP1 and P-GP was associated with a poor

Table 12.2 Expression of ABC transporters associated with cancer subtypes

Cancer	ABC transporters
Colorectal cancer	P-GP, ABCC3 (Uhlen et al. 2015)
Breast cancer	ABCG2
Lung cancer	ABCC5, ABCC3, ABCG2 (Uhlen et al. 2015)
Prostate cancer	ABCC4 (Uhlen et al. 2015)
Melanoma	ABCC1, ABCG2 (Uhlen et al. 2015)
Ovarian cancer	ABCB5, ABCA1, ABCC3 (Uhlen et al. 2015)
Pancreatic cancer	ABCA1, ABCA7 (Mohelnikova-Duchonova et al. 2013), ABCC3 (Uhlen et al. 2015)
Leukemia	ABCB5 (Yang et al. 2012)
Brain cancer	ABCC3 (Begicevic and Falasca 2017)
Liver cancer	ABCG2, ABCC3, ABCB1, ABCB5 (Uhlen et al. 2015; Begicevic and Falasca 2017)

prognosis and metastasis of cancer cells, leading to several clinical studies to determine if drug resistance could be surmounted (Shukla et al. 2008b).

Another important ABC transporter involved in drug resistance, breast cancer resistance protein (BCRP; ABCG2), was identified in 1998 when human breast carcinoma (MCF-7) cells were selected for resistance to doxorubicin in the presence of the P-GP inhibitor, verapamil. The MCF-7 cells were cross-resistant to anthracyclines, mitoxantrone, topotecan, and SN-38 (Doyle et al. 1998). This newly identified transporter, ABCG2, was highly expressed in the placenta, and the gene encoding ABCG2 was cloned and shown to efflux certain anticancer drugs out of cells (Allikmets et al. 1998; Miyake et al. 1999). ABCG2 is primarily expressed at the apical membranes of enterocytes in the intestines, human BBB capillaries, mammary glands, and hepatocytes (Cooray et al. 2002; Maliepaard et al. 2001). The ABCG2 transporter plays a significant role in protecting cells and organs from xenobiotics and protecting specific body compartments such as the placenta. It also transports folate and endogenous porphyrins, thereby maintaining folate homeostasis and protecting cells from hypoxia induced by toxic porphyrins (Ifergan et al. 2004; Krishnamurthy et al. 2004). ABCG2 exhibits overlapping substrate specificity with P-GP and MRP1. ABCG2 is a half transporter with reverse topology, i.e., the NBD followed by the transmembrane domain containing six helices. Its functional unit is a homodimer or oligomer (up to dodecamers) (Dezi et al. 2010). A summary of the most important ABC transporters that are overexpressed in different types of cancer is presented in Table 12.2.

12.3.2 Transporters as Therapeutic Targets

As discussed above, three major drug transporters, P-GP, MRP1, and ABCG2, have been implicated in the development of drug resistance in several types of cancers, as

they transport a wide range of anticancer drugs. Consequently, the pharmacological inhibition of the efflux function of these transporters was pursued as a strategy to overcome resistance to anticancer drugs in the clinic (Bugde et al. 2017).

The first generation of ABC inhibitors used in initial clinical trials as modulators of P-GP function included drugs that had already been clinically approved, such as cyclosporine A, quinine, and verapamil (Palmeira et al. 2012). These drugs inhibited the efflux function of P-GP at relatively high concentrations by competing with anticancer drugs, resulting in the use of high doses required to achieve an adequate level of these drugs within the cancer cells (Palmeira et al. 2012). However, none of these inhibitors produced an acceptable outcome, namely, surmounting drug resistance, as they produced problematic adverse effects or toxicity (Shukla et al. 2008b). For example, quinine and verapamil increased the incidence of cardiotoxicity, which prevented adequate dosing to inhibit the in vivo activity of P-GP (Arceci 1993).

The first generation of ABC transporter inhibitors was subsequently chemically modified to create a second generation of inhibitors that inhibited the activity of the ABC drug transporters at low micromolar concentrations. For example, the P-GP inhibitor PSC-833 (currently known as valspodar), a derivative of cyclosporine A, was 7- to 20-fold more potent than cyclosporine A in increasing daunorubicin retention in multidrug-resistant cancer cells (Tidefelt et al. 2000). PSC-833 lacked the immunosuppression and nephrotoxicity produced by the parent drug, cyclosporine A (Keller et al. 1992). However, these second-generation inhibitors did not produce a significant increase in efficacy (i.e., drug resistance was not overcome) when used in combination with standard anticancer drugs (van der Holt et al. 2005). Similar results were obtained in other clinical studies with PSC-833, in which its combination with an anticancer drug did not produce a beneficial clinical outcome (Greenberg et al. 2004; Lhomme et al. 2008). Furthermore, unexpected pharmacokinetic interactions occurred with the second generation of inhibitors, thereby limiting their clinical use (Coley 2010). Indeed, PSC-833 inhibited the cytochrome P-450-mediated metabolism of paclitaxel and vinblastine, resulting in high serum concentrations, increasing the frequency of toxicity in patients (Wandel et al. 1999). Similarly, VX-710 (biricodar), which was developed to treat ovarian cancer patients, also inhibited P-GP and MRP1, significantly reducing paclitaxel clearance by 24 h in phase I clinical studies (Rowinsky et al. 1998)

The third generation of ABC drug transporter inhibitors was evaluated in several preclinical models and clinical studies (Shukla et al. 2008b). This class of inhibitors was derived using combinatorial chemistry and quantitative structure-activity relationship studies to overcome the limitations of the first and second generation of inhibitors. Such inhibitors significantly decreased the efflux function of certain ABC transporters at nanomolar concentrations, thus yielding more potent and specific inhibitors. Furthermore, these compounds were postulated to have minimal pharmacokinetic interactions with other drugs, as they did not inhibit the cytochrome P450 enzymes involved in drug metabolism (Dantzig et al. 1999; Wandel et al. 1999). These inhibitors included tariquidar (XR-9576), zosuquidar (LY-335979), elacridar

(GF120918), laniquidar (R101933), mitotane (NSC-38721), and diarylimidazole (ONT-093), which were evaluated in multiple clinical trials (Kapse-Mistry et al. 2014).

Tariquidar (XR 9576) was one of the most studied third-generation inhibitors of P-GP, as it was able to reverse P-GP-mediated drug resistance (Fox and Bates 2007; Martin et al. 1999; Weidner et al. 2016). Tariquidar is well tolerated due to its selectivity and potency and had efficacy in early clinical studies, with minimal pharmacokinetic interactions with anticancer drugs (Fox and Bates 2007). However, during phase III studies, enhanced toxicity of the anticancer drugs used in combination with tariquidar occurred, and the clinical study was terminated (Fox and Bates 2007). The increase in toxicity resulted from the inhibition of P-GP in the bone marrow (stem cells) (Kannan et al. 2011). Furthermore, the lack of significant efficacy of tariquidar was also attributed to resistance mediated by transporters other than P-GP in the tumors (Nobili et al. 2006)

Elacridar (GF-120918), a dual inhibitor of P-GP and ABCG2, significantly increased the efficacy of doxorubicin and retention of paclitaxel in xenograft tumors (Kruijtzer et al. 2002). Elacridar also increased the bioavailability of certain drugs when administered orally to breast cancer patients (Kuppens et al. 2007). Zosuquidar (LY335979) was also efficacious as an inhibitor in preclinical studies (Dantzig et al. 1996). However, in clinical studies, it did not produce significant efficacy (Cripe et al. 2010; Morschhauser et al. 2007). The potential use of tariquidar and other third-generation P-GP inhibitors to modulate chemotherapy resistance is still being investigated. However, to date, no ABC transporter inhibitor alone or in combination with other chemotherapies has received approval from regulatory agencies for the treatment of drug-resistant cancers.

The studies described above primarily concerned specific inhibitors of P-GP, as it was the first ABC transporter identified. Following the discovery of the ABCG2 transporter in 1998, several groups focused their research on identifying inhibitors of that transporter to surmount drug resistance. A fungal toxin derivative, fumitremorgin C (FTC), was identified as a specific inhibitor of ABCG2 (Robey et al. 2007). However, similar to the first generation of P-GP inhibitors, it produced neurotoxicity and could not be used in patients (Rabindran et al. 2000; Allen et al. 2002). Several derivatives of FTC were later evaluated, and one such derivative, Ko143, was identified as a more potent analog of FTC that had significant efficacy in preclinical models (Allen et al. 2002). However, its clinical efficacy in surmounting drug resistance mediated by ABC2 has not been reported. Several other approved anticancer drugs known as tyrosine kinase inhibitors, including imatinib and nilotinib, were also reported to inhibit ABCG2 at low concentrations (Shukla et al. 2012). However, the clinical efficacy of tyrosine kinase inhibitors to surmount ABCG2-mediated drug resistance has not been reported in cancer patients (Shukla et al. 2012), as a majority of these drugs are transport substrates of this transporter.

Another class of compounds that inhibit ABC transporter-mediated drug resistance is natural product extracts or their active components (Wu et al. 2011a).

Nutraceuticals, originating from natural sources, are sometimes referred to as "fourth-generation inhibitors." These compounds provide one of the most diverse and novel chemical scaffolds suitable for the development of new inhibitors. Nutraceuticals such as capsaicin and piperine were found very recently to overcome cancer resistance to doxorubicin (Li et al. 2018). A combination of curcumin and piperine has been tested to increase the absorption of curcumin, which is a modulator of P-GP, ABCG2, and MRP1. The polyphenolic compounds, neochamaejasmin B (NCB), cytarabine, trabectedin, and halaven, are natural products that are potent inhibitors of ABC-mediated MDR (Li et al. 2016). A number of preclinical studies demonstrated the pharmacological advantages of using natural products to enhance the uptake of co-administered anticancer drugs by cancer cells, overcoming drug resistance (Karthikeyan and Hoti 2015; Cort and Ozben 2015; Long et al. 2016; To et al. 2017). However, a successful clinical study demonstrating the use of a natural product or extract alone or in combination with established anticancer drugs has not been reported in the literature. Further research is needed to apply high-throughput phenotypic screening and target identification methods to the discovery of natural product-based combinations of nontoxic drugs to overcome MDR.

Although researchers have not been able to exploit the potential use of the first-, second-, or third-generation inhibitors of ABC drug transporters to overcome drug resistance in the clinic, these same inhibitors, at lower doses, can be used in vivo to increase the bioavailability or enhance drug penetration across physiological barriers to the target tissue/organs.

The oral administration of certain anticancer drugs is often limited because of their permeability, which is restricted by both active and passive transport across enterocytes. The active transport of such drugs is regulated by the presence of P-GP and ABCG2, which are present on the apical side of enterocytes in the gastrointestinal tract. These transporters in the GI tract can be exploited to increase the oral bioavailability, as first demonstrated in a preclinical study in which the oral bioavailability of paclitaxel was increased from 11% in control mice to 35% in *Mdr1a*$^{-/-}$ mice (Sparreboom et al. 1997). Subsequently, several studies reported that the low oral bioavailability of P-GP substrates can be increased by the simultaneous administration of P-GP inhibitors such as cyclosporine A, valspodar, and elacridar, as shown in several preclinical and clinical studies (reviewed in Breedveld et al. (2006)). We have reported that the inhibition of intestinal ABCG2 in mice by a natural product, curcumin, increased the oral bioavailability of one of its substrates, sulfasalazine, suggesting that curcumin, a natural product modulator of P-GP and ABCG2, may be used to increase drug exposure (Shukla et al. 2011). In another preclinical in vivo study, the oral co-administration of the ABCG2 and P-GP inhibitor elacridar (GF120918) and oral topotecan, in P-GP (*Mdr1a/b*) knockout mice, produced a sixfold increase in the plasma concentration of topotecan (Jonker et al. 2000). In a clinical study, the apparent oral bioavailability increased from 40% without elacridar to 97% with elacridar (Kruijtzer et al. 2002). Overall, these data indicate that the concomitant use of P-GP and ABCG2 inhibitors with certain anticancer drugs can effectively increase the oral bioavailability of these drugs. This strategy could potentially be used in the clinic to increase the oral

bioavailability of certain anticancer drugs. Similarly, the utility of inhibiting ABC transporters at the BBB to augment brain penetration of drugs that cannot cross the BBB due to their interactions with the transporters has been reported (Hasanabady and Kalalinia 2016; Kemper et al. 2003, 2004; Drion et al. 1996; Potschka et al. 2002; Loscher and Potschka 2002; Warren et al. 2000; Hughes et al. 1998; van Vliet et al. 2006; Brandt et al. 2006; Choo et al. 2000).

12.3.2.1 ABC Transporters and Resistance to Targeted Therapies

The discovery of the tyrosine kinase inhibitor (TKI), imatinib (Gleevec), was a significant achievement in the treatment of chronic myeloid leukemia (CML) (Sacha 2014). Tyrosine kinases are essential signaling proteins that play a major role in cell development and proliferation (Gotink and Verheul 2010). Tyrosine kinases phosphorylate specific tyrosine residues in their substrates (Paul and Mukhopadhyay 2004). They can be classified as either receptor tyrosine kinases (e.g., EGFR, PDGFR, FGFR) or non-receptor tyrosine kinases (e.g., SRC, ABL, FAK) (Ségaliny et al. 2015). In addition, some of the members belong to the fibroblast growth factor receptor (FGRF) family or vascular endothelial growth factor receptor (VEGFR) family (Schlessinger 2000). Tyrosine kinases have been closely linked with cancer progression and development (Paul and Mukhopadhyay 2004). Mutations in these kinases have been identified as major mechanisms for the disruption of their biological activity. Primarily, mutations have been shown to upregulate signaling pathways that promote cell survival (Rosell et al. 2012; Flaherty et al. 2010).

Tyrosine kinase inhibitors (TKIs) are a family of several anticancer drugs that vary in their biological targets, pharmacokinetics, and toxicity profiles (Busse et al. 2001). All of the compounds share a basic mechanism of action: inhibiting a variety of tyrosine kinases (Hartmann et al. 2009). Based on the essential role of tyrosine kinases in cancer progression, developing inhibitors for these tyrosine kinases is expected to be an effective mechanism to block the upregulation of different signaling pathways, subsequently inhibiting cellular proliferation and survival (Baselga and Swain 2009; Cai 2007). Imatinib was the first TKI to be clinically approved (Sacha 2014). Other TKIs were subsequently approved, and these compounds had greater efficacy, similar adverse effects compared to imatinib, and the efficacy to surmount acquired resistance to imatinib (Agrawal et al. 2010). These TKIs included lapatinib, nilotinib, sunitinib, axitinib, apatinib, afatinib, and, recently, lenvatinib (Larson et al. 2012; Jabbour et al. 2014; Cortes et al. 2012). One TKI usually blocks multiple kinases. For example, vandetanib inhibits VEGFR, EGFR, and RET kinases (Wells and Santoro 2009). However, according to the primary enzyme that TKIs block, they can be divided into the following subfamilies: BCR-ABL inhibitors (e.g., imatinib, nilotinib), epidermal growth factor receptor inhibitors (e.g., lapatinib, gefitinib), and inhibitors of the vascular endothelial growth factor receptors (e.g., sunitinib, sorafenib) (Arora and Scholar 2005).

Dacomitinib (Guo et al. 2018) and quizartinib (Li et al. 2017) are the most recent TKIs that have been characterized. The second-, third-, and fourth-generation TKIs (e.g., the epidermal growth factor receptor (EGFR) TKIs) vary in their chemical structures and their potencies in wild-type or cancer cells containing T790 M or C797S mutations (Wu and Fu 2018). The TKIs act primarily by competing with the binding of ATP to the intracellular catalytic domain of tyrosine kinase, which consequently inhibits cross-phosphorylation of various biological proteins that are crucial for regulating downstream signaling pathways (Busse et al. 2001). Ultimately, the TKIs arrest cell growth and proliferation. Although TKIs have been clinically successful in treating various cancers, resistance to the TKIs has been reported (Chen and Fu 2011), which reduces the overall efficacy of these targeted therapies (Chen and Fu 2011). A number of mechanisms can produce TKI resistance, and the overexpression of ABC transporters is a confirmed mechanism (Deng et al. 2014). Certain ABC drug transporters, especially P-GP, ABCG2, MRP4, and MRP7, have been implicated in resistance to TKI chemotherapy, as most of the TKIs are efflux substrates of ABC transporters at low concentrations (Brozik et al. 2011). For example, imatinib was found to interact with both P-GP and ABCG2 transporters (Shukla et al. 2008a). TKI concentrations for cancer patient treatments are usually low (nanomolar or picomolar potencies), making TKIs susceptible to efflux by specific ABC transporters (Shukla et al. 2012). However, TKIs at higher concentrations may be effective inhibitors of ABC transporters (Shukla et al. 2012). They bind to important ABC transporters such as ABCB1 and ABCG2 in the substrate-binding pocket in the transmembrane region, not at the ATP sites as do TKs, and result in the inhibition of their efflux function. TKIs such as imatinib, nilotinib, gefitinib, erlotinib, cediranib, apatinib, and vandetanib have been reported to reverse multidrug resistance mediated by certain ABC transporters (Mi et al. 2010).

According to Wu and Fu (Wu and Fu 2018), the specific mechanisms that mediate TKI-induced reversal of the MDR phenotype are not clear because there are insufficient studies describing the structure-activity relationships between TKIs and ABC transporters. One possible mechanism by which TKIs modulate ABC transporters is by interacting with the substrate-binding sites of these transporters, which is different from the ATP-binding site of TKIs (Mi et al. 2010). It has been reported that the TKIs subsequently interfere with the transport function of the ABC transporters, with no effect on the expression level of these transporters (Mi et al. 2010).

12.3.2.2 Examples of Resistance to Combination Therapy

While TKIs can be used as monotherapies in the clinic, they can also be employed in combination with adjuvant therapies to surmount drug resistance to various chemotherapeutic regimens (Wang et al. 2014). Data indicate that TKIs have greater selectivity and lower toxicity compared to other nontargeted chemotherapies (Wang et al. 2014; Wu and Fu 2018). The results of clinical trials evaluating combination therapy in patients with MDR were summarized by Wu and Fu

(2018). These combination therapies, such as erlotinib + gemcitabine, lapatinib + capecitabine, nintedanib + docetaxel, and erlotinib + carboplatin, were used in clinical trials to treat pancreatic, breast, and non-small cell lung and ovarian cancers, respectively.

Possible benefits of the combination of TKIs with other chemotherapeutic drugs were determined in several cell and animal models with high levels of MDR (Shen et al. 2009b; Mi et al. 2010). For example, nilotinib, a BCR-Abl tyrosine kinase inhibitor, significantly potentiated the cytotoxicity (i.e., efficacy) of several chemotherapeutic drugs that were substrates for transporters such as ABCG2 and P-GP (Tiwari et al. 2009). Nilotinib significantly increased the intracellular accumulation of paclitaxel and mitoxantrone in ABCB1- and ABCG2-overexpressing cells, respectively (Tiwari et al. 2009). The combination of apatinib with mitoxantrone resulted in significant reversal of multidrug resistance in wild-type HEK293/ABCG2-R2 cells transfected with the ABCG2 transporter (Mi et al. 2010). Apatinib (5 μM) also reversed ABCB1-mediated multidrug resistance to doxorubicin and mitoxantrone in transfected HEK293/ABCB1 cells (Mi et al. 2010). The reversal efficacy of apatinib occurs primarily through direct binding and inhibition of the ABCG2 and P-GP efflux functions, thus increasing the intracellular accumulation of chemotherapeutic drugs in cancer cells (Mi et al. 2010). In another study, IM and nilotinib (2.5 μM) significantly reversed drug resistance mediated by the MRP7 transporter and restored the sensitivity of MRP7-overexpressing cells (HEKMRP7-2) to paclitaxel and vincristine. The efficacy of imatinib and nilotinib was primarily mediated by inhibition of the MRP7 transport function (Shen et al. 2009b). Also, neither compound significantly altered the expression of MRP7 (Shen et al. 2009b). Lapatinib and erlotinib at 0.625, 1.25, and 2.5 μM concentrations, similar to imatinib and nilotinib, significantly increased the intracellular accumulation of paclitaxel in MRP7-overexpressing cells, reversing MDR (Kuang et al. 2010). The TKI sunitinib also significantly reversed ABCG2-mediated MDR, with no significant effect on the efflux function of the P-GP or ABCC1 transporters (Dai et al. 2009). Overall, the above findings indicate that TKIs could be used to reverse MDR mediated by several ABC transporters. These compounds can be combined with other anticancer drugs to restore the efficacy of certain anticancer drugs and prevent treatment failure.

12.3.3 Explanations for the Lack of Efficacy of ABC Transporter Inhibitors

Despite preclinical and clinical data demonstrating that the inhibition of ABC transporters could be a viable strategy to surmount drug resistance, to date, no inhibitors are being used in the clinic to treat drug-resistant tumors. There are several possible reasons for this.

One of the major hurdles that has precluded the success of the transporter inhibitor approach could be the functional expression of transporters in organs

such as the liver and kidneys that are also responsible for determining the pharmacokinetic profile of anticancer drugs. The inhibition of drug transporter activity resulted in significant drug-drug interactions, leading to a decrease in systemic clearance (for drugs that are hepatically metabolized and renally excreted). This led to an increase in toxicity, which occurred in many clinical studies. In general, this problem would apply to even the most specific ABC transporter inhibitors, as specific inhibition of even one transporter can lead to increased drug levels in many tissues because ABC transporters are present at physiological barriers throughout the body. Consequently, these issues need be taken into consideration with any attempt to develop ABC transporter inhibitors for future clinical use.

Furthermore, during these clinical studies, it was assumed that the ABC drug transporters were uniformly expressed in the tumors. However, their expression in the tumors was not determined at the time of enrollment in the clinical study. Consequently, a certain threshold of P-GP or ABCG2 expression in a tumor biopsy should have been a criterion for patient enrollment in the clinical study evaluating ABC drug transporter inhibitors, as these inhibitors were supposed to produce efficacy by competitively inhibiting the efflux function of ABC transporters, which accounts for the drug-resistant phenotype. This was supported by several clinical studies that reported the correlation of ABC transporter expression with malignant progression and a more aggressive phenotype (Hlavata et al. 2012; Johnatty et al. 2008). Another major factor in the failure of the "inhibitor strategy" in the clinic could be the overlapping substrate specificity between ABC drug transporters, which provides functional redundancy. The relative expression of these drug transporters is a dynamic process that is controlled by physiological processes that could change from underexpression to overexpression, resulting in significant variability when the transporter is inhibited in patients using combination chemotherapy. In addition, as members of the ABCB and ABCC families have various roles in the immune system (Lai and Gallo 2009), inhibition of their function by competitive inhibitors may have led to impaired antitumor immune responses, resulting in the loss of efficacy for the combination treatment.

12.4 Advanced Strategies to Overcome MDR

As mentioned above, the effort to develop four generations of ABC transporter inhibitors was focused on compounds that bind to the ABC transporters and block their active sites. These strategies did not result in identifying a clinically successful compound to overcome cancer MDR. Thus, current research is shifting toward novel strategies to overcome the ABC transporter-mediated resistance by mechanisms other than blocking the substrate-binding site (Li et al. 2016).

MicroRNA (miRNA) interference is one of the new approaches to inhibit ABC transporters at the translational level through their binding and inhibition of transporter mRNA. Several miRNAs, such as miR-27a,-451, -296, -298, and -1253, were found to inhibit P-GP-mediated MDR in breast cancer cells (Bao et al. 2012; Hong

et al. 2010). Based on these findings, a significant number of synthetic siRNAs are being developed to inhibit ABC transporter genes (Zhang et al. 2015). Another strategy is the development and optimization of monoclonal antibodies targeting ABC transporters, which would enhance the anticancer efficacy of chemotherapeutic drugs. Antibodies such as MRK-16, MRK-17, and UIC2 significantly inhibited P-GP function in in vitro and in vivo models, particularly when used with another P-GP inhibitor (Mechetner and Roninson 1992; Naito et al. 1993). A recombinant, single-chain Fv (scFv) antibody was isolated using the phage display approach, and this antibody selectively targeted the extracellular domain of the MRP1 transporter without affecting other MRP members or other ABC transporters (Binyamin et al. 2004). This selective targeting of MRP1 can be utilized to reverse MDR in tumors overexpressing the MRP1 transporter (Binyamin et al. 2004).

Recently, gene therapy to overcome MDR has been shown to be a viable approach (Lage 2016). However, studies with gene manipulation to minimize or abolish drug resistance are limited to in vitro systems and have not progressed sufficiently to in vivo systems. The development of RNA-guided clustered regularly interspaced short palindromic repeats (CRISPR), and CRISPR-associated Cas9-gene editing technology may provide an excellent tool for the precise regulation of gene expression in eukaryotic cells (Gilbert et al. 2013). For example, the restoration of chemosensitivity ($\geq$ 50%) to doxorubicin was achieved in adriamycin-resistant (A2780/ADR) ovarian cancer cells by knocking down ABCB1 gene expression (Norouzi-Barough et al. 2018). A significant decrease in P-GP gene expression was associated with increased sensitivity of the transfected cells to doxorubicin with single-guided RNAs (sgRNAs) (Norouzi-Barough et al. 2018). The CRISPR-Cas9 gene editing tool was also used to silence the expression of the ABCB1 gene in Madin-Darby canine kidney II (MDCK) cells (Simoff et al. 2016). It has been reported that doxorubicin produced significant cytotoxicity in drug-resistant breast cancer cells pretreated with the RNA-guided endonuclease (RGEN) system, whereas no toxicity occurred in untreated cells (Simoff et al. 2016). The potency of doxorubicin was significantly increased in the cells treated with the protein-RNA complex, as well as in those treated with plasmids, suggesting that mutation of the *mdr1* gene by intracellular delivery of the Cas9-sgRNA complex increased the efficacy of doxorubicin (Ha et al. 2016). Therefore, the Cas9-mediated disruption of a drug resistance-related gene may be a promising way to counteract MDR in cancer cells. However, whether the application of such technology to edit cancer genes in patients would be beneficial is not yet known (Cai et al. 2016).

Nanoparticles loaded with a combination of anticancer drugs is also one of the strategies being investigated to surmount MDR by increasing the anticancer drug stability, cellular delivery, bypassing the efflux by ABC drug transporters and specific distribution (Gandhi et al. 2014). One example of this approach is the use of paclitaxel iron oxide nanoparticles (PTX-NPs). When the PTX-NPs are combined with anti-ABCG2 monoclonal antibody (mAb), a significant reduction in the volume of the multiple myeloma (MM) tumor was observed, with a consequential increase in the survival rate in vivo when injected into non-obese diabetic/severe combined immunodeficiency mice containing the MM tumor (Yang et al. 2014). Another

example is the loading of an RNA interference-chemotherapeutic drug combination in a nanocarrier to target the gene expression of P-GP and increase the delivery of the chemotherapeutic drug, as well as its efficacy (Gandhi et al. 2014). Polymeric nanoparticles, liposomal nanoparticles, micelles, and gold nanoparticles are other examples of nanocarriers being utilized to surmount ABC transporter-mediated MDR in cancer (Xu et al. 2006; Chen et al. 2009; Jin et al. 2015). The co-delivery of doxorubicin and siRNA Bcl2 to target P-GP-mediated MDR by mesoporous nanoparticles significantly increased the intracellular concentration of doxorubicin (Chen et al. 2009). Polymer-lipid (P123-DOPE) supported mesoporous silica nanoparticles (PLS-MSNs) represent another nanocarrier combination (Zhang et al. 2014). Lipid-coated silica nanoparticles prevent the escape of the encapsulated anticancer drugs (irinotecan/CPT-11) before reaching the target cells. Upon encountering the acidic pH environment of a tumor cell, the anticancer drug is released. The lipid polymer was also found to inhibit BCRP-mediated CPT-11 efflux in drug-resistant MCF-7/BCRP breast cancer cells (Zhang et al. 2014). Moreover, the CPT-11-loaded PLS-MSNs were less toxic in BALB/c nude mice bearing drug-resistant breast tumors (Zhang et al. 2014). The use of a nanodrug delivery system demonstrated that nanodrugs can be efficiently delivered to the nuclei to effectively kill cancer cells (Qiu et al. 2015).

The biomaterial cyclodextrin, which removes cholesterol, was used by Shi and co-workers to reverse MDR (Shi et al. 2015). Acetylated α-cyclodextrin (Ac-aced), when inside a cell, releases α-cyclodextrin molecules due to hydrolysis of Ac-aced in an acidic subcellular organelle (Shi et al. 2015). The pH-responsive nanoparticle (NP), derived from acetylated α-cyclodextrin (Ac-aCD), is endocytosed by MDR cancer cells, and intracellularly transported by endo-lysosomal compartments, which ultimately increases the efficacy of paclitaxel, docetaxel, cis-diamminedichloroplatinum, camptothecin, and doxorubicin by targeting P-GP expression and its ATPase activity (Shi et al. 2015).

Zhu and co-workers proposed that low-density lipoprotein may be an efficient vector in targeting tumor cells in which low-density lipoprotein (LDL) was isolated from human plasma and loaded with cholesterol-conjugated siRNA (Chol-siRNA) to silence the *mdr1* gene of the tumors (Zhu et al. 2014). Chol-siRNA/LDL-coupled N-succinyl chitosan nanoparticles loaded with doxorubicin (Dox-siRNA/LDL-SCS-NPs) were more easily taken up by the tumor cells compared to those without the lipoprotein, resulting in a higher uptake efficiency of siRNA and, subsequently, a decrease in P-GP mRNA expression.

Chemical modifications of anticancer drugs can also be used to facilitate the uptake of anticancer drugs into multidrug-resistant cancer cells, hence increasing the accumulation of the drugs in the cells and their cytotoxicity (Ganju et al. 2014). Common delivery systems include poly(lactic-co-glycolic acid) (PLGA)-based nanoparticles. Further in situ modification can be made to the PLGA nanoparticle's surface with human serum albumin (HSA). The attached HSA hydrophilic chains increase the cellular uptake by receptor-mediated endocytosis (Manoochehri et al. 2013). As a result, there is a greater uptake of the anticancer drugs by the cell. The bound HSA PLGA nanoparticle, which encapsulates the drug, significantly

increased cytotoxicity compared to the free drug after 72 h of incubation (Manoochehri et al. 2013). Similar effects can also be attained by coating PLGA nanoparticles with some Food and Drug Administration (FDA)-approved "Generally Recognized As Safe" (GRAS) substances. GRAS substances include natural polymers, polyethylene glycol and its derivatives, and pluronic polymers, which have been reported to inhibit ABC transporters (Sosnik 2013). Tao et al. synthesized a PLGA and TPGS polymer that increased the solubility of docetaxel, allowing for greater permeability through the cell membrane (Tao et al. 2013). The TPGS polymer inhibited P-GP and increased the accumulation of docetaxel, overcoming MDR in cancer cells.

The use of combination therapies utilizing a chemosensitizer concurrently with a chemotherapeutic agent can enable synergism between chemical entities, bringing about a higher chance of killing resistant cancer cells. Guo and co-workers (Guo et al. 2016) found a novel nanoparticular pre-chemosensitizer which can be used as a nanocarrier for chemotherapy drugs. The same group then went on to improve on the delivery system to create a new self-assembled formula of amphiphilic poly (curcumin-dithiodipropionic acid)-b-poly(ethylene glycol)-biotin nanoparticulate system which can achieve targeted delivery to cancer cells and, remarkably, degrade at intracellular concentrations of glutathione and then release both curcumin and doxorubicin, which functions as a chemosensitizer, and the chemotherapeutic, respectively.

One strategy to overcome MDR was the use of aptamers to target resistant cancer tumors. Aptamers are small (usually 20–60 nucleotides), single-stranded RNA or DNA oligonucleotides that bind target molecules with high affinity and specificity (Zhou et al., Darmostuk et al. 2015). Soldevilla and co-workers (Soldevilla et al. 2016) synthesized the first aptamer to target resistant tumors that overexpressed MRP1 through a novel approach known as systematic evolution of ligands by exponential enrichment (SELEX). This technique selects for aptamers by first having an RNA library and then screening the library against the MRP1 aptatope peptide for hits, coupled with counterselection using control peptides (Soldevilla et al. 2016). With the SELEX technique, a MRP1-CD28 bivalent aptamer can have dual effects, binding to MRP1-expressing resistant tumors while simultaneously delivering CD28 co-stimulatory signal to T lymphocytes (Soldevilla et al. 2016). The success of this novel bioengineering approach has been shown in melanoma-bearing mice, in which there is reduced tumor growth and prolonged survival (Soldevilla et al. 2016).

The use of aptamers for cancer therapy is also described by Qiu and co-workers, who used DNA self-assembly to develop a nanodrug system carried by a cell-targeted near-infrared (NIR)-responsive nanotruck to treat drug-resistant cancer. Nanodrug therapy involves small drug-loaded gold nanoparticles (carriers) that can self-assemble onto the side face of a silver-gold nanorod (NR, or nanotruck), with end faces modified by a cell type-specific internalizing aptamer (Qiu et al. 2015).

Recently, the concept of exploiting the energy source of a cell to surmount MDR has gained attention. Wang and co-workers (2018) reported that lipid membrane-coated silica-carbon (LSC) hybrid nanoparticles, which target the mitochondrial pyruvate pathway, result in the production of reactive oxygen species (ROS) under the illumination of near-infrared (NIR) radiation. NADH is oxidized into NAD^+ by the ROS. Consequently, ATP is not available for use by the ABC transporters. When LSC nanoparticles were used together with NIR laser irradiation in vivo, the expression of ABC transporters was significantly reduced, while the distribution of these pumps within a cell was conversely increased. Therefore, MDR could be overcome by encapsulating anticancer drugs in LSC nanoparticles followed by their delivery to target cancer cells, which produce ROS, or to develop drugs that modulate ROS directly to target MDR cells. However, these strategies must be utilized together with NIR radiation.

Another technology that was developed is transkingdom RNAi (tkRNAi). With this technology, a nonpathogenic bacteria such as *E.coli* is used to deliver short hairpin RNA molecules into resistant cancer cells, ultimately resulting in effective RNA interference (Kruhn et al. 2009; Lage and Kruhn 2010). The anti-ABCB1 shRNA expression vector bearing *E. coli* resulted in significant inhibition of the expression of P-GP in several types of human cancer cells and a subsequent increase in chemotherapeutic drug concentrations (Kruhn et al. 2009).

Finally, targeting ABC-mediated drug resistance via photo-destruction technology may represent another potential approach. This technique involves producing an aggregation of drug, sequestering ABCG2 extracellular vesicles at the surface of resistant cancer cells which are then targeted by illumination (Goler-Baron and Assaraf 2012). This selective illumination results in the production of reactive oxygen species, destruction of these vesicles, lysis of resistant tumor cells, and reversal of MDR without significantly affecting normal epithelial cells (Goler-Baron and Assaraf 2012). A summary and representative example of strategies that have been suggested and investigated to overcome ABC transporter-mediated MDR are presented in Table 12.3.

Table 12.3 Strategies to overcome ABC transporter-mediated MDR

Inhibiting the expression of MDR transporters	Inhibiting the efflux function of MDR transporters
Specific polypeptide transcriptional repressor: (5-azacytidine, HMN-176), MDR1 (Efferth et al. 2001; Tanaka et al. 2003)	Efflux inhibitors that are not substrates (Borowski et al. 2005)
MicroRNA interference (miRNA): (mir-298, mir296, etc.) (Bao et al. 2012; Hong et al. 2010)	Competitive MDR inhibitors (substrates): first, second, third generation (Borowski et al. 2005)
Anti-MDR ribozymes: (Wang et al. 2003)	Cytostatic anticancer agents with faster influx than efflux (Borowski et al. 2005)
Post translational medication: inhibitors of transporter phosphorylation (Conseil et al. 2001)	Nanoparticle technology: paclitaxel iron oxide nanoparticles (Yang et al. 2014)
Advance gene knockdown technology: CRISPR-Cas9 (Norouzi-Barough et al. 2018)	Nutraceuticals (capsaicin and piperine) (Li et al. 2018)
Targeting mitochondrial ROS (LSC nanoparticles) (Wang and Gao 2018)	

12.5 Conclusions and Perspectives

Although ABC transporters play an essential role in the development of multidrug-resistant cancer, efforts that have been made to target these transporters have not yet been successful. Future approaches to overcoming multidrug resistance will most likely (1) use bioinformatics for personalized medicine, (2) consistently probe for novel mechanisms linking ABC transporters and diseases, including MDR, and (3) accelerate the discovery of anticancer drugs by using high-throughput platforms, as well as formulating either novel or existing chemotherapeutics as nanomedicines to increase drug delivery to resistant cancer cells. Table 12.4 demonstrates approaches that can simultaneously be used to target MDR mediated by ABC transporters.

Furthermore, with enhanced understanding through pharmacogenomics and genome-wide association studies, which demonstrate an association between an individual's response to drugs at the genetic level and variation at the cellular or physiological level, the role of ABC transporters in the context of cancer MDR can be better elucidated by specifically identifying SNPs for specific ABC transporter genes which would have a higher propensity to produce the MDR phenotype. Thus, the response of an individual to chemotherapeutic drugs will be predicted, and personalized dosing can be utilized to improve chemotherapy.

Finally, by applying novel formulations and advanced technology as strategies to target ABC transporters and multidrug resistance, the delivery of anticancer drugs to resistant cells will be maximized. Nanoparticles, monoclonal antibodies, and gene technologies are examples of these strategies. Combination therapies to maximize chemosensitivity should also be further investigated. Such approaches may hold the key to overcoming the ever-evolving problem of drug-resistant cancer.

Table 12.4 Approaches that can exploit ABC transporters to overcome MDR mediated by ABC transporters (with representative examples)

1	Targeting ABC transporters that are overexpressed in tumors (to account for variations in expression profile of transporters) (Sun et al. 2012; Wu et al. 2008)
2	Targeting transcription factors that specifically regulate the overexpression of ABC drug transporters in target tumors (significant differences were detected between mRNA and protein levels) (Marquez and Van Bambeke 2011)
3	Using high-affinity modulators of ABC drug transporters (TKIs such as nilotinib, imatinib, and gefitinib have high affinity the transporters) (Brendel et al. 2007; Shukla et al. 2012; Ozvegy-Laczka et al. 2004)
4	Targeting transporters only in tumors and not interfering with normal physiological functions of these transporters in normal tissues (detoxification, drug deposition, and immunological functions in different organs) (Choi and Yu 2014; Robey et al. 2010)
5	Nontoxic ABC transporter modulators that have synergistic activity and can be used prophylactically to prevent the incidence of MDR (TKIs and other modulators were investigated to increase sensitivity of tumor to other cytotoxic agents (Anreddy et al. 2014; Shukla et al. 2012; Shen et al. 2009b; Kuang et al. 2010; Mi et al. 2010)

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