Review Article

Mechanism of multidrug recognition by MDR1/ABCB1

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MDR1/ABCB1, a member of the ABC group of proteins, is clinically important because it is not only involved in multidrug resistance in cancer but also affects the pharmacokinetic properties of various drugs. The most puzzling feature of MDR1 is that it recognizes and transports such a wide variety of substrates. In the present review, the function of MDR1 is compared with that of other ABC proteins, particularly MDR2/ABCB4, to understand the mechanism of drug recognition and transport by MDR1. MDR2, the amino acid sequence of which has 86% similarity to that of MDR1, excretes phosphatidylcholine and cholesterol in the presence of bile salts. ABCA1 transfers phospholipids, preferentially phosphatidylcholine, and cholesterol to lipid-free apoA-I to generate pre-β-HDL, and ABCG1 excretes phospholipids, preferentially sphingomyelin, and cholesterol. Cholesterol also binds directly to MDR1 and modulates substrate recognition by MDR1. Cholesterol may fill the empty space of the drug-binding site and aid the recognition of small drugs, and facilitates the ability of MDR1 to recognize compounds with various structures and molecular weights. Eukaryote ABC proteins may retain similar substrate binding pockets and move bound substrates in an ATP-dependent manner. The prototype of eukaryote ABC proteins might be those involved in membrane lipid transport. (*Cancer Sci* 2007; 98: 1303–1310)

Multidrug resistance 1 (MDR1/ABCB1) is a plasma membrane-located glycoprotein that confers multidrug resistance to cancer cells by actively excreting structurally diverse chemotherapeutic compounds from cells.[1–3] MDR1 is clinically important because it not only confers multidrug resistance but also affects the pharmacokinetic properties of various drugs.[4–6]

MDR1 is a 1280-amino acid protein with two symmetrical halves connected by a short linker region.[7] Each half consists of six putative transmembrane helices (TM) followed by a nucleotide binding fold (NBF), in which ATP is hydrolyzed to energize the transport (Fig. 1). Hydrolysis is thought to be directly linked to drug transport and both NBF should be catalytically active,[8,9] although the exact number of ATP molecules hydrolyzed in a single transport is still obscure.[10,11]

The *MDR2/ABCB4* gene (also called *MDR3* in humans) exists next to *MDR1* on human chromosome 7q. MDR2 is a 1279-amino acid protein and has an amino acid sequence with 76% identity and 86% similarity to that of MDR1. As the chromosomal region containing *MDR1* and *MDR2* genes is often amplified in multidrug-resistant cell lines, *MDR2* is also expected to be involved in multidrug resistance against anticancer drugs; however, the overexpression of *MDR2* scarcely confers drug resistance. Instead, *mdr2* knockout mice manifested an unexpected phenotype, the absence of phosphatidylcholine (PC) in bile, suggesting that MDR2 excretes phospholipids rather than xenobiotics into bile;[12] however, the mechanism of phospholipid efflux mediated by MDR2 has not been characterized in detail.

Although the true clinical relevance of multidrug resistance is still heavily debated, MDR1 is believed to be one of the key molecules that causes multidrug resistance in cancer. The most puzzling feature of MDR1 is that it recognizes and transports such a wide variety of substrates (Fig. 2), and many efforts have been made to understand the mechanism. In the present review, we compare the function of MDR1 with that of other ABC proteins, particularly MDR2, to understand the mechanism of drug recognition and transport by MDR1.

Discovery of *MDR1* gene as a multidrug transporter

The emergence and outgrowth of the population of tumor cells resistant to multiple anticancer drugs is a major obstacle in cancer chemotherapy. One form of resistance is characterized by the energy-dependent removal of a variety of structurally unrelated cytotoxic agents by membrane transporter proteins.[13] Human *MDR1* cDNA was isolated from a multidrug-resistant KB carcinoma cell line, KB-C2.5, selected for its resistance to colchicine, in 1986,[2,7] and turned out to code for P-glycoprotein,[14] a surface glycoprotein reported to be overexpressed in drug-resistant Chinese hamster ovary cell mutants.[1] The overexpression of human and mouse P-glycoprotein conferred resistance to multiple drugs such as *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, and taxol.[2,15,16] A few years later, human *MDR1* was isolated from the adrenal and it was found that cDNA, isolated from KB-C2.5, was associated with a Gly-to-Val substitution at position 185, in the predicted cytoplasmic loop between TM2 and TM3.[17] This mutation increased colchicine resistance and decreased vinblastine resistance.[17,18]

The first of the eukaryote ABC proteins

MDR1 was discovered as the first of the eukaryote ABC proteins.[7,19] Now we know that 48 or 49 ABC protein genes exist on human chromosomes.[20] One major characteristic feature of ABC proteins is that defects in their functions are related to various diseases (Table 1). ABC proteins can be classified into seven subgroups (A to G) based on amino acid sequences of the ATP-binding domain (Fig. 1). For example, mutations in the *ABCA1* gene cause Tangier disease, or familial HDL deficiency.[21–23] Defects in ABCA3 cause fatal surfactant deficiency in newborns,[24] and mutations in the *ABCA4* gene, specifically expressed in photoreceptors, cause macular retinal dystrophy.[25] Defects either of ABCG5 or ABCG8 increase plant sterol concentration in plasma and cause sitosterolemia.[26]

Three different types of functions of membrane proteins

Another interesting feature of the ABC protein family is that its members exhibit three different types of functions of membrane

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Human ABC (ATP Binding Cassette) proteins

A	B	C	D	E	F	G
ABCA1	ABCB1(MDR1)	ABCC1(MRP1)	ABCD1(ALDP)	ABCE1	ABCF1	ABCG1
ABCA2	ABCB2(TAP1)	ABCC2(MRP2)	ABCD2		ABCF2	ABCG2
ABCA3	ABCB3(TAP2)	ABCC3	ABCD3(PMP70)		ABCF3	ABCG4
ABCA4	ABCB4(MDR2)	ABCC4	ABCD4			ABCG5
ABCA5	ABCB5	ABCC5				ABCG8
ABCA6	ABCB6	ABCC6				
ABCA7	ABCB7	ABCC7(CFTR)				
ABCA8	ABCB8	ABCC8(SUR1)				
ABCA9	ABCB9	ABCC9(SUR2)				
ABCA10	ABCB10	ABCC10				
ABCA12	ABCB11(BSEP)	ABCC11				
ABCA13		ABCC12				
		ABCC13				

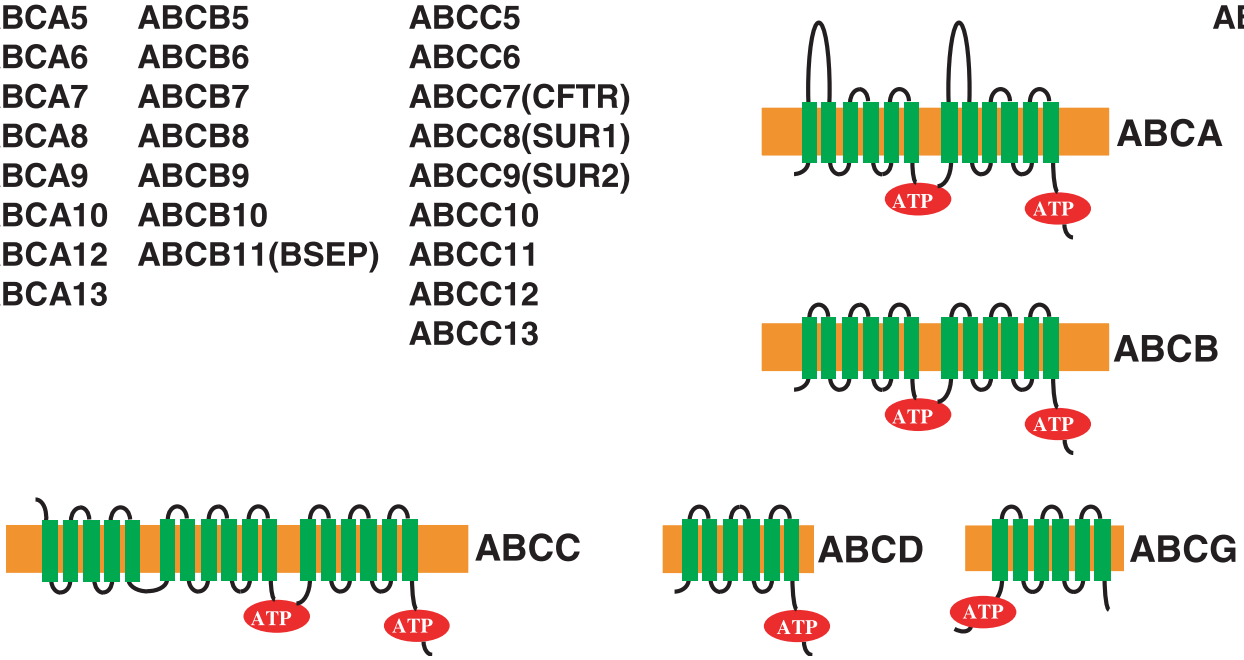

Fig. 1. Members of each subfamily (A to G) of human ABC proteins and their typical secondary structures.

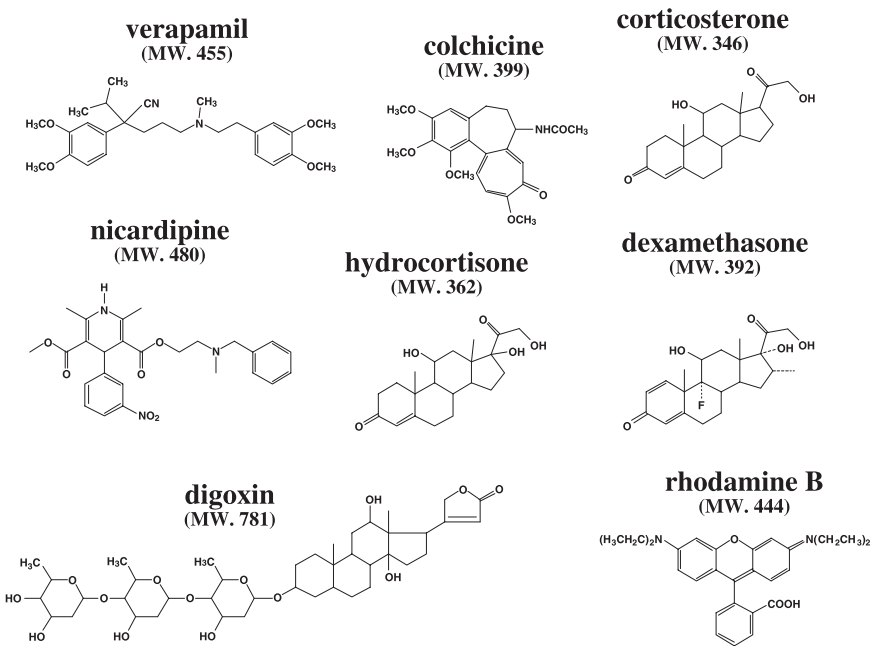

Fig. 2. Structure and molecular weight of typical substrates transported by human ABCB1/MDR1.

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Table 1. Human ABC proteins, functions and diseases

Symbol	Gene	Amino acid	Function/phenotype, disease
ABCA subfamily			
ABC1	ABCA1	2261	Cholesterol and phospholipid efflux/HDL deficiency
ABC2	ABCA2	2436	Lipid transport?/Abnormal myelin formation
ABC3	ABCA3	1704	Secretion of pulmonary surfactant/Surfactant deficiency in newborn
ABCR	ABCA4	2273	Retinoic acid transport/Stargardt disease 1
ABCA7	ABCA7	2146	Phospholipid transport
ABCA12	ABCA12	2595	Lipid transport?/Harlequin ichthyosis
ABCB subfamily			
MDR1, PGY1	ABCB1	1280	Xenobiotics efflux pump/Multidrug resistance in cancer
TAP1	ABCB2	808	Transport of antigen peptide into ER lumen/Behçet's disease
TAP2	ABCB3	653	Transport of antigen peptide into ER lumen/Behçet's disease
MDR2/3	ABCB4	1279	Secretion of phosphatidylcholine into bile/Intrahepatic cholestasis
ABCB6	ABCB6	842	Transport of porphyrin in mitochondria
ABC7	ABCB7	752	Transport of iron-sulfate complexes in mitochondria
SPGP, BSEP	ABCB11	1321	Bile acid export/Intrahepatic cholestasis
ABCC subfamily			
MRP1	ABCC1	1531	Transport of detoxificated xenobiotics/multidrug resistance in cancer
MRP2/cMOAT	ABCC2	1545	Bilirubin export/Dubin–Johnson syndrome
MRP3	ABCC3	1527	Excretion of sulfate and glucuronide metabolites
MRP4	ABCC4	1325	Transport of nucleoside based anti-viral drugs and prostaglandin
MRP5	ABCC5	1437	Transport of cyclic nucleotides and nucleoside monophosphate analogs
MRP6	ABCC6	1503	?/pseudoxanthoma elasticum
CFTR	ABCC7	1480	Cl^- channel/Cystic fibrosis
SUR1	ABCC8	1581	ATP sensitive K^+ channel regulator in pancreatic β-cells/PHHI
SUR2	ABCC9	1549	ATP sensitive K^+ channel regulator in cardiac myocyte
ABCD subfamily			
ALDP	ABCD1	745	Peroxysomal transport of very long fatty acid/Adrenoleukodystrophy
ALDR	ABCD2	740	Peroxysomal transport of very long fatty acid/Adrenoleukodystrophy
PMP70	ABCD3	659	Peroxysomal transport of very long fatty acid
ABCG subfamily			
ABCG1	ABCG1	678	Transport of cholesterol and phospholipid
ABCP, BCRP	ABCG2	655	Multidrug resistance, specific expression in stem cells
ABCG5	ABCG5	651	Export of phytosterols/sitosterolemia
ABCG8	ABCG8	673	Export of phytosterols/sitosterolemia

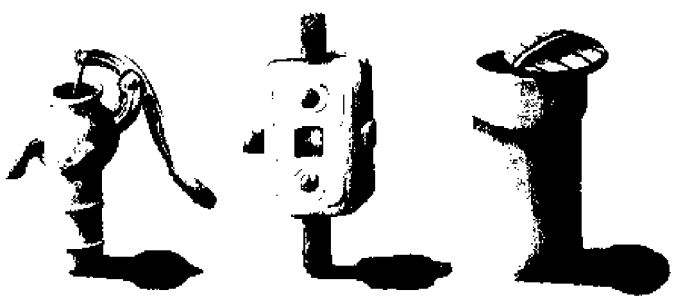

Fig. 3. Drawings representing the three types of ABC proteins.

proteins (channel, receptor, and transporter), despite being very much alike in their predicted secondary structures. The images in Fig. 3 represent transporters, channels, and receptors to show how these three groups of membrane proteins differ in their functions.[27]

Cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7) is the only ABC protein clearly proven to function as a channel.[28–30] CFTR is a voltage-independent Cl^- channel found in the epithelial cells of many tissues, and plays a major role in regulating Cl^- flux. The role of ATP binding or hydrolysis on channel gating is not clear yet. ATP or ADP could alter the open probability by reflecting the intracellular metabolic state like SUR1. Mutations in CFTR cause cystic fibrosis, one of the most common serious diseases, which affects 1 in 2000–2500 people in northern Europe and the USA. In addition to its Cl^- channel activity, CFTR is also suggested to act as a regulator of both an outwardly rectifying Cl^- channel (ORCC) and an epithelial Na^+ channel. Numerous abnormalities in cystic fibrosis are believed to be related to this multifunctionality of CFTR.[31]

The sulfonylurea receptor (SUR1/ABCC8) was identified as a target protein for sulfonylureas, such as glibenclamide, which is most commonly used in the treatment of non-insulin-dependent diabetes mellitus.[32] SUR1 is a subunit of the pancreatic β-cell K_{ATP} channel, which is a hetero-octamer composed of pore-forming Kir6.2 subunits and SUR1 that co-assembles with 4:4 stoichiometry,[33–35] and plays a key role in the regulation of glucose-induced insulin secretion. SUR1 is suggested not to be a channel or transporter itself, but to be a switch that regulates the opening and closing of Kir6.2 channel subunits by monitoring the intracellular metabolic state, particularly ADP concentration.[27,36,37]

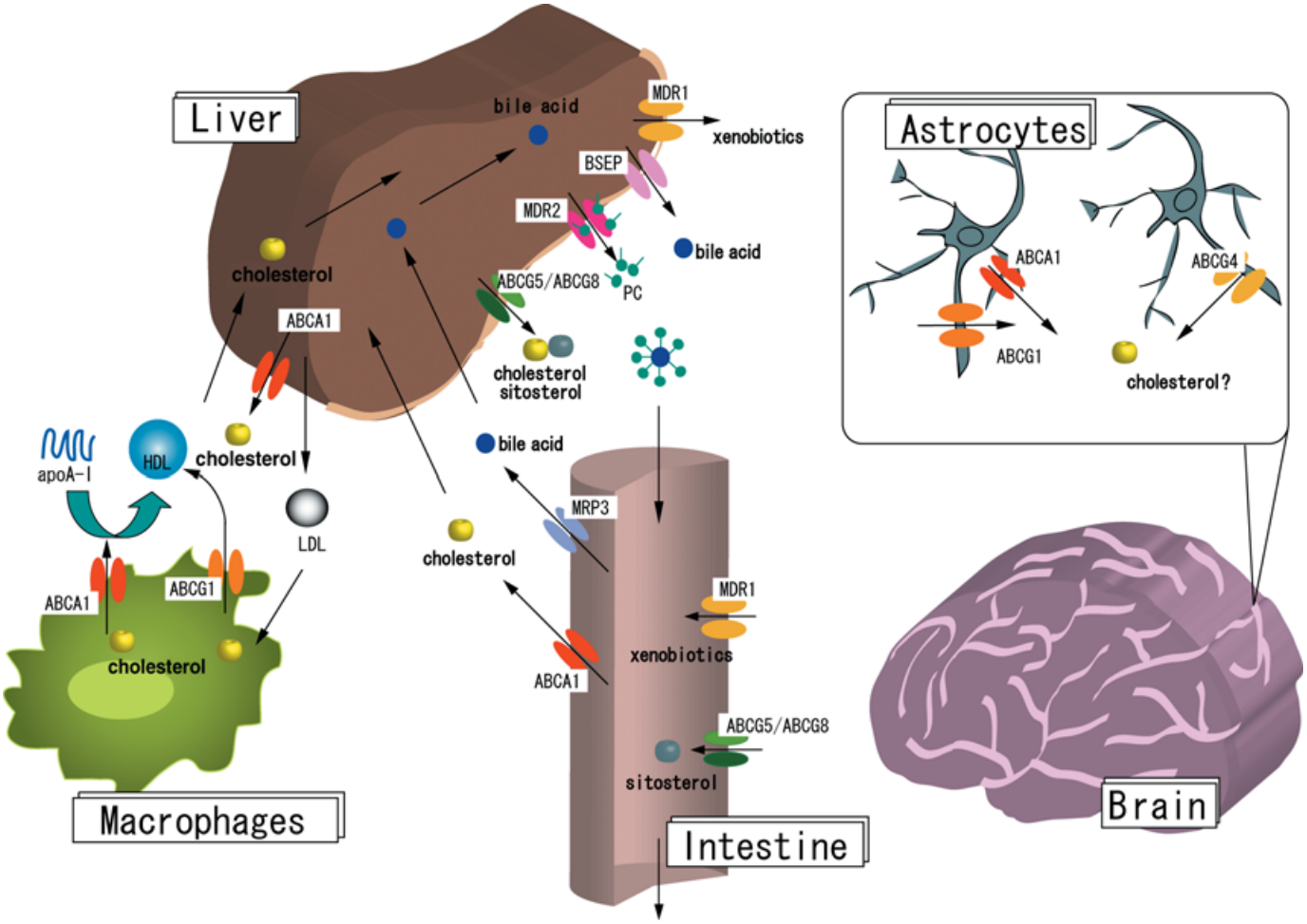

Fig. 4. Human ABC proteins transport lipids and xenobiotics in various tissues.

Most of the other eukaryotic ABC proteins seem to be active transporters, in which ATP hydrolysis provides the free energy necessary to transport substrates from one side of the membrane to the other, although there are still many members with unknown functions. All the eukaryotic transporter-type ABC proteins studied to date transport substrates outwardly from cytosol into organelles or out of cells. One exception may be a plant MDR-type ABC protein, CjMDR1, which is involved in alkaloid transport in *Coptis japonica*, a perennial medicinal plant.[38] It is proposed that CjMDR1 is involved in the translocation of berberine from the root to the rhizome, and functions as an influx pump for berberine.

ABC proteins in lipid homeostasis

ABC proteins have been generally recognized as drug efflux pumps that protect the body from various toxic substances. *MRP1/ABCC1* was isolated from multidrug-resistant cancer cells[39] shortly after *MDR1*. MRP1 transports various organic anionic conjugates, including glutathione, glucuronide, and sulfate conjugates, while MDR1 transports lipophilic compounds in unmodified forms. The discovery of MDR1 and MRP1 had a strong impact on the field of cancer chemotherapy and pharmacodynamics, and may be one of the reasons for the strong impression that ABC proteins function as drug transporters; however, recent findings have suggested that their physiological role as self-defense machinery against xenobiotics is only one side of the importance of ABC proteins.

Membrane lipids, various phospholipids and cholesterol, move among organelles in cells and also move from the liver to various tissues and are excreted into the canalicular and intestinal lumen. Many ABC proteins have been found to be involved in these pathways (Fig. 4). MDR2 and ABCB11 are involved in bile formation as transporters for PC and bile salts.[12,40] ABCA1 is required for HDL generation.[41] ABCA2 may be involved in lipid movement to generate the myelin sheath.[42] ABCA3 is involved in the biogenesis of lamellar body-like structures, in which pulmonary surfactant, consistent with phospholipids and cholesterol, is stored.[43] ABCG5 and ABCG8 excrete plant sterols into the canalicular lumen.[26]

MDR2 in bile formation

The function of MDR2 is required for proper bile formation. Mice with homozygous disruption of the *mdr2* gene show almost complete absence of PC from their bile,[12,44–46] which causes segmental biliary strictures due to periductal fibrosis, fibro-obliteration of bile ducts, and spontaneous gallstone formation.[46,47] Human *MDR2* mutations result in a wide spectrum of phenotypes, ranging from progressive familial intrahepatic cholestasis type 3 to adult cholestatic liver disorders characterized by elevated γ-glutamyl transpeptidase levels.[48] The primary function of biliary phospholipid excretion is suggested to be to protect cell membranes facing the biliary tree against bile salts, and PC in bile salt-mixed micelles reduces the detergent activity of micelles.[49]

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It has been suggested that MDR2 promotes the transfer of PC from the inner to the outer leaflet of the plasma membrane, mainly based on experiments using a fluorescent PC analog containing a 7-nitro-2,1,3-benzoxadiazol group (NBD);[50,51] however, MDR1, which is not physiologically involved in PC secretion into bile, also translocates a short-chain PC analog (C6-NBD-PC).[51] Smith *et al*. demonstrated that, in fibroblasts from transgenic mice expressing the human *MDR2* gene, MDR2 translocates long-chain radioactive PC that is exchanged by PC-transfer protein shuttling between the outer leaflet of the plasma membrane and acceptor liposomes in the medium.[52] This experiment suggested that liposomes themselves were unable to accept long-chain PC translocated by MDR2 and some acceptors such as PC-transfer proteins might be required for the excretion of PC to the medium. Thus, the mechanism of PC secretion mediated by MDR2 has not yet been clarified.

Bile salt-dependent efflux of cellular phospholipids by MDR2

The function of MDR1 and MDR2 was compared using the HEK293 cell line stably expressing these proteins. Phospholipids (preferentially PC) and cholesterol were secreted from HEK293/MDR2 cells in the presence of bile salts.[53] As the excretion of phospholipids and cholesterol was not observed with MDR2-K435M or MDR2-K1075M, in which the Walker A lysine in either NBF was substituted by methionine, MDR2 mediates lipid efflux in an ATP-dependent manner, like other ABC transporters; however, no lipid secretion was observed from HEK293 cells expressing MDR1, even in the presence of bile salts.

No lipid secretion was observed from HEK293/MDR2 cells in the absence of bile salts, but the presence of NaTC below the cmc promoted the lipid efflux mediated by MDR2.[53] These results suggest that the monomer form of bile salts most likely functions, at least initially, in supporting MDR2-mediated lipid efflux. The orders of bile salt-induced efflux of phospholipids and cholesterol, from highest to lowest, were as follows: taurocholate (NaTC) > glycocholate (NaGC) > cholate (NaC), which was not consistent with the order of maximum solubility of cholesterol in bile salt micelles, NaGC > NaTC > NaC.[54] The hydrophilic–hydrophobic balance of bile salt has been quantified as the bile salt monomeric hydrophobicity index.[55,56] Hydrophobicity indices, from least to most hydrophobic, are NaTC (0) < NaGC (+0.07) < NaC (+0.13);[55] therefore, the bile salt-dependent efflux of phospholipids and cholesterol by MDR2 was inversely correlated with the bile salt hydrophobicity index. It is possible that bile salt monomers may associate not only with the phospholipid secreted by MDR2 but also with amino acids lining the surface of the cavity of MDR2, and that this helps the release of phospholipid.

Drug recognition by MDR2

MDR2 has a 76% overall amino acid sequence identity with MDR1,[57] therefore, it is conceivable that MDR2 has conserved domains for substrate recognition in common with MDR1. Indeed, it has been reported that both MDR1 and MDR2 confer resistance to aureobasidin A, an antifungal cyclic depsipeptide antibiotic, when expressed in yeast.[58] The resistance of yeast cells to aureobasidin A conferred by MDR2 can be overcome by vinblastine, verapamil, and cyclosporine A. Transepithelial transport of C6-NBD-PC and digoxin by MDR2 through LLC-PK1 cells is inhibited by vinblastine, verapamil, and cyclosporin A, indicating an interaction between these compounds and MDR2.[59] The translocation of NBD-PC in yeast secretory vesicles is mediated by both MDR1 and MDR2 and abrogated by verapamil.[50] It was showed that verapamil almost completely abolished the NaTC-dependent efflux of phospholipids and cholesterol mediated by MDR2.[53] These results indicate that MDR1 and MDR2 have quite similar substrate-binding domains; however, MDR1 cannot transport PC even in the presence of NaTC, whereas MDR2 can.

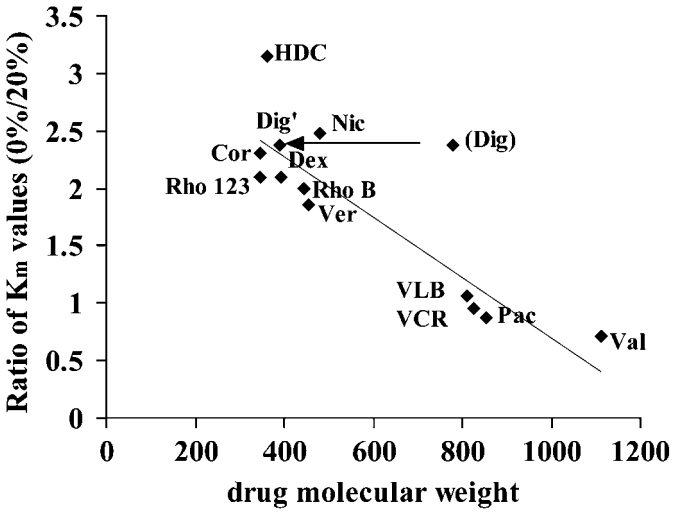

Fig. 5. Relationship between the ratio of K_m values in the absence and presence of 20% (w/w) cholesterol and molecular weights of drugs. The line represents the best fit linear regression ($r^2 = 0.8075$) except the points for digoxin. Rho123, rhodamine 123; Cor, corticosterone; Dex, dexamethasone; Dig; digoxin; Dig′, aglycon form of digoxin; HDC, hydrocortisone; Nic, nicardipine; Pac, paclitaxel; RhoB, rhodamine B; Val, valinomycin; VCR, vincristine; Ver, verapamil; VLB, vinblastine.

Modulation of drug-stimulated ATPase activity of MDR1 by cholesterol

As described above, MDR2 mediates the excretion of PC and cholesterol in the presence of bile salt monomers in an ATP-dependent manner; therefore, it is conceivable that MDR1 also interacts with membrane lipids. Indeed, it has been reported that cholesterol stimulates basal (i.e. without any drugs) ATPase activity,[60,61] and that cholesterol is recognized and transported as an endogenous substrate of MDR1.[62] It was also shown that the depletion of cholesterol reduced the transport activity of MDR1, resulting in the intracellular accumulation of drugs in cells.[61,63,64]

The ATPase activity of MDR1 was analyzed using purified human MDR1 reconstituted in liposomes containing 0–20% (w/w) cholesterol.[65] Interestingly, cholesterol affected not only basal ATPase activity but also the drug-stimulated ATPase activity of MDR1. The effects of cholesterol on K_m were drug-specific. When the ratio of the K_m of each drug in the absence and presence of 20% cholesterol was plotted versus the molecular weight (MW) of that drug, a strong correlation was found between them (Fig. 5). At first glance, digoxin did not fit the correlation, but when the aglycon form of digoxin (MW: 390) was plotted on the graph, it fitted well. The binding affinity of drugs, such as rhodamine 123, dexamethasone, verapamil, nicardipine, digoxin, corticosterone, hydrocortisone, and rhodamine B, with a low MW, between 350 and 500, increased in the presence of 20% cholesterol. The binding affinity of drugs, such as vinblastine, vincristine, and paclitaxel, with an MW of between 800 and 900, was not much affected by cholesterol. The binding affinity of valinomycin, the MW of which is more than 1000, decreased in the presence of 20% cholesterol.

Model for drug recognition by MDR1

The strong correlation between the effect of cholesterol on K_m for drugs and their MW suggests that the primary effect of

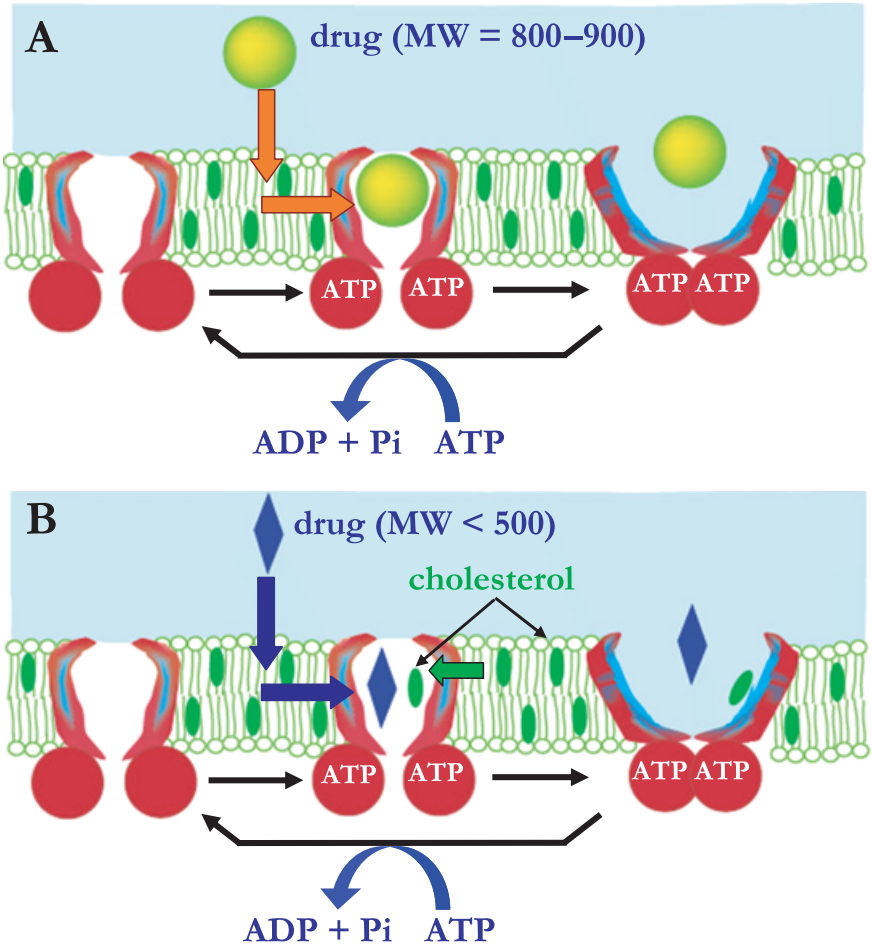

Fig. 6. Model for drug recognition mechanism by ABCB1/MDR1.

cholesterol could be on the drug-binding site. MDR1 possesses multiple drug-binding sites[66–68] and these sites are located in the middle of the lipid bilayer.[69] Shapiro *et al*. demonstrated that MDR1 possesses at least three positively cooperating drug-binding sites,[67] an H site selective for Hoechst 33342 and colchicine, an R site selective for rhodamine 123 and anthracyclines, and another site for progesterone. Drug binding to one site stimulates transport by the other. Moreover, rhodamine 123 and progesterone in combination stimulate the transport of Hoechst 33342 in an additive manner. Martin *et al*. also assigned four drug-binding sites, three of which were classified as sites for transport and one for the regulation of transport.[66] Cholesterol may directly bind to or allosterically affect the drug-binding site to adjust its size to the drug (Fig. 6). As the binding affinity of drugs with an MW of between 800 and 900 (vinblastine, vincristine, and paclitaxel) is not affected by the presence of cholesterol (Fig. 6A), the drug-binding site of MDR1 may best fit drugs of these sizes. When small drugs, with an MW of 350–500, bind to MDR1, cholesterol (MW: 386.7) may fill the empty space or allosterically tighten the drug-binding site and help the recognition of smaller drugs (Fig. 6B).

It has been demonstrated that the bulkiness of side chains at the position of His61 and its neighboring amino acid residues in TM1 is important for substrate specificity.[70,71] For example, the replacement of His61 by amino acids with bulkier side chains increased resistance to small drugs such as colchicine and VP16, while it lowered resistance to a large drug, vinblastine. Recently, it was also suggested that TM1 forms part of the drug-binding pocket by cross-linking experiments using the thiol-reactive analog of verapamil.[72] These observations also suggest that the size of the drug-binding pocket is important for recognizing drugs.

Comparison of the function of MDR1 and MDR2 with other ABC proteins

ABCA1 transfers phospholipids and cholesterol to lipid-free apoA-I to generate pre-β-HDL.[41] Purified ABCA1 shows high ATPase activity when reconstituted in liposomes made of synthetic PC or sphingomyelin (SM), suggesting that ABCA1 recognizes phospholipids with choline head groups;[73] however, when cellular SM content is reduced, apoA-I-dependent cholesterol efflux by ABCA1 increases. These results suggest that ABCA1 preferentially transports PC and cholesterol probably from non-raft domains.[74] In contrast, ABCG1 preferentially secretes SM and cholesterol.[75] ABCG1 may recognize cholesterol with SM as substrates to transport (Fig. 7). Alternatively, ABCG1-mediated cholesterol efflux occurs in raft domains.

As described in the present review, MDR2 excretes PC and cholesterol, and cholesterol may directly bind to the drug-binding site of the drug transporter MDR1 to help the recognition of smaller drugs, although MDR1 does not transport cholesterol even in the presence of bile salts. The shape of the substrate binding sites of ABC proteins could be conserved to recognize cholesterol in the membrane during evolution. These results suggest that the prototype of eukaryote ABC proteins was

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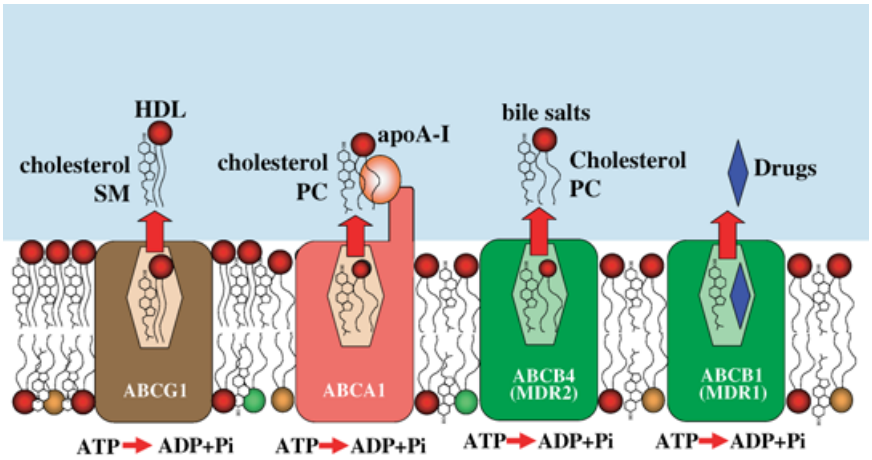

Fig. 7. Comparison of the function of ABCG1, ABCA1, ABCB4/MDR2, and ABCB1/MDR1. ABCA1 transfers phospholipids and cholesterol to lipid-free apoA-I to generate pre-β-HDL. ABCG1 secretes sphingomyelin (SM) and cholesterol to HDL. ABCB4/MDR2 excretes phosphatidylcholine (PC) and cholesterol in the presence of bile salts. Cholesterol may directly bind to the drug-binding site of ABCB1/MDR1 and help the recognition of smaller drugs, but cholesterol is not secreted.

involved in membrane lipid transport, but not in drug efflux. It would be rational to consider that ABC proteins involved in lipid homeostasis are also primary transporters just like MDR1, and recognize the PL–cholesterol complex as a substrate to transport. Further studies are needed to verify these models, but this study facilitates our understanding of the mechanism of multidrug recognition by MDR1.

Acknowledgments

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