Editorial

ABC Transporters: Role in Modulation of Drug Pharmacokinetics and in Physiopathology and Therapeutic Perspectives

The superfamily of ABC transporters comprises 49 proteins in humans. They all use ATP hydrolysis as an energy source and share common structural features. In particular, the presence of 2 membrane-spanning domains made of 6 transmembrane segments and of 2 ATP binding cassettes is required to make them functional. Yet, the function of many of these proteins still needs to be elucidated and their substrates remain unknown, but for those that are best characterized, it is clear that they play a critical role in the maintenance of cell homeostasis, by modulating the cell concentration in xenobiotics or in physiological substrates.

In the present issue, we will examine the role of selected transporters in this superfamily, to illustrate their importance in drug disposition or in physiopathology. We will also discuss therapeutic perspectives related to modulation of their activity. The figure illustrates the transporters we will focus on, together with the indication of their function and of the pathology associated to their dysfunction.

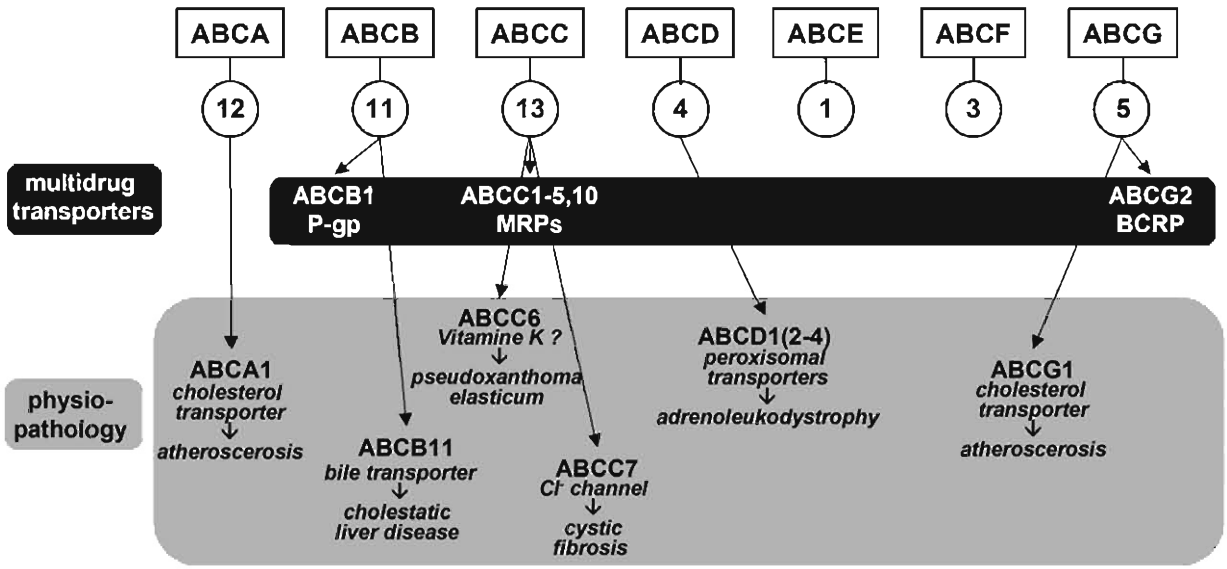


Figure: Families of ABC transporters (squared) with indication of the number of transporters in the family (circled).

Xenobiotic transporters catalyse the efflux of drugs, or metabolites thereof, out of the cells. In contrast to many other transporters, they all display very broad substrate specificity, recognizing their substrates based on physico-chemical properties rather than on specific molecular determinants, hence their appellation of 'Multidrug Transporters'. These transporters belong to 3 families, namely ABCB for P-glycoprotein, ABCC for MRPs (Multidrug-resistance Related Proteins), and ABCG for the half transporter BCRP (Breast Cancer Resistance Protein).

In this issue, we will review their specific role in modulating drug pharmacokinetics [1] or in conferring resistance to anticancer chemotherapy [2], and the interest of inhibitors to reverse their effect. We will also discuss for 2 of them (ABCB1 and ABCC2) how genetic polymorphisms can lead to variations in their transport capacity among individuals and therefore justify therapeutic monitoring of blood levels, or patient's genotyping for drugs with narrow therapeutic index [3].

Other ABC transporters rather transport physiological substrates that can be highly variable in their chemical nature (Chlorure ions for ABCC7 or cholesterol for ABCA1, for example). The physiological role of these transporters has been generally identified when discovering the molecular mechanism of the disease their defect can cause, which may explain why for most of them, it remains largely unknown.

In this issue, we will describe how the dysfunction in ABCA1 or ABCG1, ABCB11, ABCC6, ABCC7, or ABCD transporters can lead to atherosclerosis [4], cholestatic liver disease [5], Pseudoxanthoma elasticum [6], Cystic Fibrosis [7], or adrenoleukodystrophy [8], respectively. We will also examine the strategies that are developed to correct these defects in a therapeutic perspective.

Françoise Van Bambeke
(*Guest Editor*)

Pharmacologie cellulaire et moléculaire
Louvain Drug Research Institute
Université catholique de Louvain
Brussels, Belgium
E-mail: francoise.vanbambeke@uclouvain.be

REFERENCES

[1] Marquez B, Van Bambeke F. ABC multidrug transporters: target for modulation of drug pharmacokinetics and drug-drug interactions. Curr Drug Target 2011; 12(5): 600-20.
[2] Shukla S, Ohnuma S, Ambudkar SV. Improving cancer chemotherapy with modulators of ABC drug transporters. Curr Drug Target 2011; 12(5): 621-30.
[3] Haufroid V. Genetic polymorphisms of ATP-binding cassette transporters ABCB1 and ABCC2 and their impact on drug disposition. Curr Drug Target 2011; 12(5): 631-46.
[4] Ye D, Lammers B, Zhao Y, Meurs I, Van Berkel TJC, Van Eck M. ATP-binding cassette transporters A1 and G1, HDL metabolism, cholesterol efflux, and inflammation: important targets for the treatment of atherosclerosis. Curr Drug Target 2011; 12(5): 647-60.
[5] Stieger B, Beuers U. The canalicular bile salt export pump BSEP (ABCB11) as a potential therapeutic target. Curr Drug Target 2011; 12(5): 661-70.
[6] Váradi A, Szabó Z, Pomozi V, de Boussac H, Füllöp K, Arányi T. ABCC6 as a target in pseudoxanthoma elasticum. Curr Drug Target 2011; 12(5): 671-82.
[7] Amaral MD. Targeting CFTR: how to treat cystic fibrosis by CFTR-repairing therapies. Curr Drug Target 2011; 12(5): 683-93.
[8] Morita M, Shimozawa N, Kashiwayama Y, Suzuki Y, Imanaka T. ABC subfamily D proteins and very long chain fatty acid metabolism as novel targets in adrenoleukodystrophy. Curr Drug Target 2011; 12(5): 694-706.

ABC Multidrug Transporters: Target for Modulation of Drug Pharmacokinetics and Drug-Drug Interactions

Béatrice Marquez and Françoise Van Bambeke*

Pharmacologie Cellulaire et moléculaire, Louvain Drug Research Institute, Université Catholique de Louvain, B-1200 Brussels, Belgium

Abstract: Nine proteins of the ABC superfamily (P-glycoprotein, 7 MRPs and BCRP) are involved in multidrug transport. Being localised at the surface of endothelial or epithelial cells, they expel drugs back to the external medium (if located at the apical side [P-glycoprotein, BCRP, MRP2, MRP4 in the kidney]) or to the blood (if located at the basolateral side [MRP1, MRP3, MRP4, MRP5]), modulating thereby their absorption, distribution, and elimination. In the CNS, most transporters are oriented to expel drugs to the blood. Transporters also cooperate with Phase I/Phase II metabolism enzymes by eliminating drug metabolites. Their major features are (i) their capacity to recognize drugs belonging to unrelated pharmacological classes, and (ii) their redundancy, a single molecule being possibly substrate for different transporters. This ensures an efficient protection of the body against invasion by xenobiotics. Competition for transport is now characterized as a mechanism of interaction between co-administered drugs, one molecule limiting the transport of the other, which potentially affects bioavailability, distribution, and/or elimination. Again, this mechanism reinforces drug interactions mediated by cytochrome P450 inhibition, as many substrates of P-glycoprotein and CYP3A4 are common. Induction of the expression of genes coding for MDR transporters is another mechanism of drug interaction, which could affect all drug substrates of the up-regulated transporter. Overexpression of MDR transporters confers resistance to anticancer agents and other therapies. All together, these data justify why studying drug active transport should be part of the evaluation of new drugs, as recently recommended by the FDA.

Keywords: P-glycoprotein**,** BCRP, MRP, ADME properties, drug-drug interactions.

INTRODUCTION

The proteins from the ATP-binding cassette (ABC) transporters superfamily share as common features a capacity to actively transport molecules through the membranes, and to use ATP hydrolysis as an energy source. They have been classified in seven subfamilies (ABCA to ABCG), according mainly to sequence homologies and structural organization [1]. The topology and nomenclature of ABC transporters have been extensively reviewed elsewhere [2-4] and will not be addressed here.

Most of the 48 human ABC transporters (without the truncated ABCC13 with still unknown function [5]) play a role in the export of physiological substrates (amino acids, peptides, lipids, inorganic ions…), but nine of them are rather associated to a Multi-Drug Resistance (MDR) phenotype, due to their ability to extrude out of the cells a large variety of xenobiotics.[1] These are the P-glycoprotein (ABCB1, P-gp), the Multidrug Resistance associated Proteins or MRPs (MRP1-MRP7, also referred to as ABCC1-6 and ABCC10), and the Breast Cancer Resistance Protein or BCRP (ABCG2). In addition, the intracellular transporter ABCA3 has also been implicated in multidrug resistance in leukemia cells, as it can sequester drugs inside lysosomes [8]. The role of these MDR transporters, and of P-gp in particular, is well described in the context of resistance to anticancer drugs [9, 10]. Yet, as they are widely distributed in the organism [11], they also play an important role in the modulation of absorption, tissue distribution and elimination of their substrates or in the protection of sanctuaries, like the central nervous system (Fig. **1**). MDR ABC transporters are therefore considered as a major intervenient in the pharmacokinetics of many drugs, which can in its turn modulate their pharmacological activity or their toxicity [12-14]. A first goal of this paper is to review the current knowledge on the role of MDR ABC transporters in drug transport and its consequences in terms of ADME properties.

A striking characteristic of these MDR transporters is the wide variety of apparently non chemically-related substrates they can accommodate. This is not yet fully understood, but the structure of the murine P-gp (Abcb1a) recently resolved at a 3.8 Å resolution [15], together with the structural models of different MDR ABC established by homology modeling using crystallographic structures from bacterial homologs [16-22], may be helpful in this respect. A pharmacological consequence of this broad substrate specificity is that co-administration of drug substrates may cause drug-drug interactions by competition for a same transporter. Moreover, drugs can also induce the expression of transporters, modifying thereby their capacity to transport their substrates [13]. A second goal of this paper is to examine how these recently described mechanisms of drug-drug interactions can affect drug pharmacokinetic properties.

*Address correspondence to this author at the Unité de Pharmacologie Cellulaire et moléculaire, UCL 7370 Avenue Mounier 73, B-1200 Brussels, Belgium; Tel: +32-2-764-73-78; Fax: +32-2-764-73-73;
E-mail: francoise.vanbambeke@uclouvain.be

[1] Transporters involved in drug influx belong to another superfamily of transporters, namely the SLC (Solute-Linked Carrier) family (a family of secondary transporters that comprises the organic anion transporting polypeptides (OATPs), the organic anion transporters (OATs) or the organic cation transporters (OCTs)). These also play an important role in drug pharmacokinetics and drug-drug interactions [6, 7] but will not be discussed here.

1389-4501/11 $58.00+.00 © 2011 Bentham Science Publishers Ltd.

PHYSIOLOGICAL FUNCTIONS OF MDR ABC TRANSPORTERS

Table **1** illustrates the localization, expression levels (at the mRNA or protein level), and physiological substrates of MDR ABC transporters. Caution is required however when data refers only to mRNA levels, as discrepancies between mRNA and protein levels may exist. For example, BCRP expression in kidney is low at mRNA level but higher at protein level [23]. Moreover, spliced mRNA variants do not always code for an entire, functional protein [24-27]. While some of these transporters, like P-gp, have a very broad tissue distribution, others are expressed only in a few organs, like MRP7. For the latter, this suggests specific roles in these organs, even though these have rarely been evidenced. Considering MRP7, for example, it is interesting to note that it is expressed in the heart and can transport leukotriene C4, a well known vasoconstrictor agent [28]. Likewise, MRP4 is highly expressed in prostate and expels cyclic nucleotides that control erectile function and smooth muscle activity in the urinary tract [29]. The expression level of a given transporter can also markedly vary from one organ to the other, depending of its specific role. P-gp for example is highly expressed at the apical membrane of many epithelial cells (enterocytes, renal tubules, canalicular membrane of hepatocytes) or brain capillary endothelium [11], in relation with its detoxification function. More intriguingly, some transporters can be found either at the apical or at the basolateral membrane, depending on the tissue. This is mostly the case for MRP4, which is usually located at the basolateral membrane but is found at the apical surface of renal epithelial cells and brain endothelial cells (for a review, refer to [30]). The basolateral transporters MRP1 and MRP5 have also been detected at the apical membrane of brain endothelial cells [31], although at low levels. This may contribute to reinforce the protective effect of P-gp or BCRP on the brain.

MDR TRANSPORTERS AND MODULATION OF DRUG PHARMACOKINETICS

Fig. (**1**) illustrates the main role of MDR ABC transporters with respect to drug disposition in the organism. Those that are localized at the apical surface of the cells bordering the elimination organs will contribute to cell detoxification by expelling xenobiotics into the bile, the urine or the faeces; those that are expressed at the basolateral surface will rather contribute to drug (re)absorption by driving them from the intracellular medium to the blood [30, 32]. MRPs mainly transport Phase II metabolites (drug conjugates to glutathione, glucuronate or sulfate [33]) and constitute therefore the "Phase III" of drug elimination [34, 35].

At the level of barriers separating the blood from sanctuaries or vulnerable organs like the brain, the placenta or the testis, most transporters are oriented towards a transport from the organ to the blood, as a way to protect these fragile sites from foreign invasion [36, 37]. This role is best evidenced by the specific neurotoxicity of ivermectin in beagle dogs that are naturally deficient in P-glycoprotein [38]. In non-polarized cells, efflux pumps can contribute to reduce the cellular concentration of drugs and hence, their pharmacological activity if they act upon an intracellular target. This is well exemplified by the reduction in intracellular activity of fluoroquinolones, macrolides, or daptomycin against bacteria infecting macrophages expressing MDR transporters [39, 40] or of anti-HIV drugs in infected macrophages and lymphocytes [41]. *A fortiori*, overexpression of MDR transporters is a well established mechanism of resistance of cancer cells to chemotherapy [10, 42, 43].

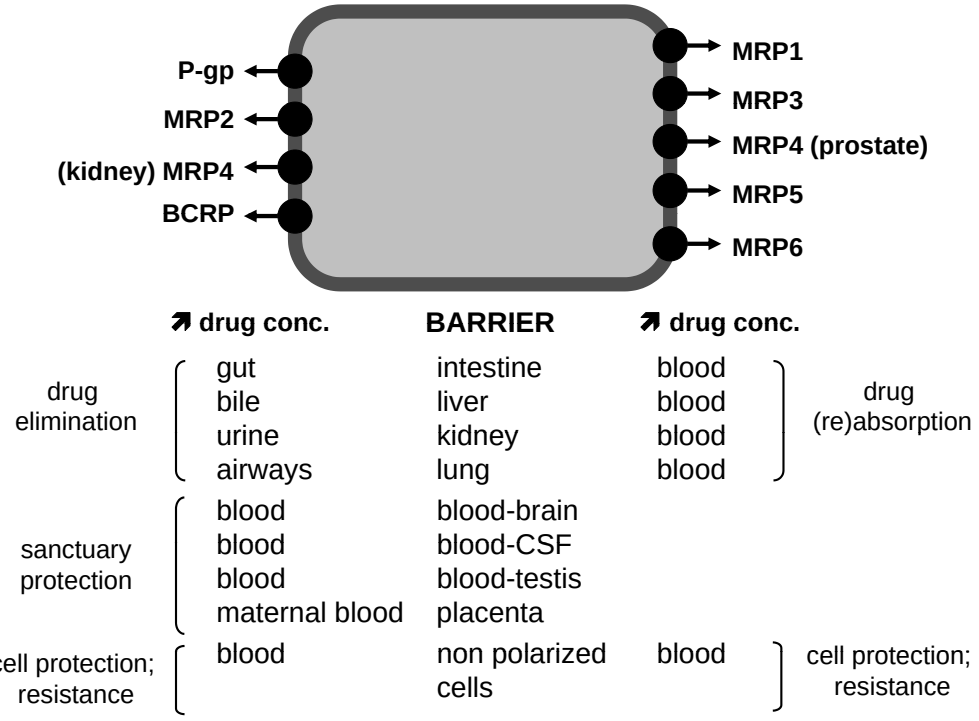


Fig. (1). Illustration of the role of MDR ABC transporters in the modulation of drug disposition when expressed at the apical or basolateral side of the cells bordering the main barriers in the body, or in non polarized cells.

Table 1. Localization and Physiological Substrates of ABC Transporters

ABC Transporter	Localization	Tissue Distribution		Physiological Substrates	Refs.
		High Expression	Low Expression		
P-gp (ABCB1)	Apical	Kidney, adrenal gland, liver, pancreas, intestine, lung, blood-brain barrier, placenta	Prostate, skin, heart, skeletal muscle, ovary	Phospholipids, cytokines, steroids	[11, 220]
MRP1 (ABCC1)	Basolateral (except in placenta and BBB)	Kidney, lung, testis, skeletal and cardiac muscles, placenta (apical)	Liver, intestine, brain	Glutathione, glutathione conjugates (LTC4, DNP-SG), bilirubin glucuronides, bile salts	[31, 221-223]
MRP2 (ABCC2)	Apical	Liver, kidney, small intestine, placenta		LTC4, DNP-SG, bilirubin glucuronides, sulphated bile salts	[183, 222, 224, 225]
MRP3 (ABCC3)	Basolateral	Adrenal gland, intestine, pancreas, gallbladder, placenta	Liver, kidney, prostate*	Bile salts, LTC4, estradiol-17β-glucuronide, bilirubin-glucuronides	[222, 226, 227]
MRP4 (ABCC4)	Apical (kidney, BBB) or basolateral (prostate, choroid plexus)	Prostate	Ovary*, testis*, kidney, lung*, intestine*, liver, brain, pancreas	cAMP, cGMP, bile salts, folate, conjugated steroids, prostaglandins (PGE1, PGE2)	[31, 228-232]
MRP5 (ABCC5)	Basolateral (except in BBB)	Skeletal* and cardiac muscle, testis*	Brain, neurons, liver*	cAMP, cGMP, folate, DNP-SG	[31, 227]
MRP6 (ABCC6)	Basolateral	Liver, kidney, skin, lung, heart, intestine, pancreas, stomach		LTC4, DNP-SG	[233, 234]
MRP7 (ABCC10)	?		Colon*, skin*, testis*, pancreas*	Estradiol-17β-glucuronide, LTC4	[235, 236]
BCRP (ABCG2)	Apical	Placenta, breast, blood-brain barrier, liver, intestine,	Kidney, lung, ovary*, testis* pancreas*	Vitamins (riboflavin, biotin), porphyrins, estrogen sulfate conjugates	[23, 237-240]

BBB, blood-brain barrier; LTC4, leukotriene C4; DNP-SG, 2,4-dinitrophenyl-*S*-glutathione.
*Data for gene expression only (mRNA); otherwise, the tissue distribution refers to protein detection.

Table 2. Pharmacologically Relevant Substrates of MDR ABC Transporters

ATC Code		Pharmacological Class	Drug	P-gp	MRP1	MRP2	MRP3	MRP4	MRP5	MRP6	MRP7	BCRP	Refs.
Alimentary tract and metabolism													
A	02B	H2-receptor antagonists	Cimetidine	+								+	[241, 242]
			Nizatidine	+									[243]
			Ranitidine	+									[242]
	03F	Propulsives	Domperidone	+									[101]
	04A	Antiemetics	Ondansetron	+									[101]
	07D	Antipropulsives	Loperamide	+									[101]
Cardiovascular system													
C	01A	Cardiac glycosides	Digoxin	+*								-*	[100, 241]
			Talinolol	+ (r)									[244]
	01B	Anti-arrhythmics	Quinidine	+ (r)*									
	02C	Antihypertensives	Prazosin	+ (m)								+ (m)	[245]
	07A	Beta-blocking agents	Celiprolol	+									[246]
	08C	Calcium channel blockers (vascular)	Nicardipine	*								+	[247, 248]
			Nifedipine	+(r)*								+	[247, 249]
	08D	Calcium channel blockers (cardiac)	Diltiazem	+*									[250]
			Verapamil	+*									[251]
	09C	Angiotensin II antagonists	Losartan	+	-	-							[252]

(Table 2) Contd…..

ATC Code		Pharmacological Class	Drug	P-gp	MRP1	MRP2	MRP3	MRP4	MRP5	MRP6	MRP7	BCRP	Refs.
C	10A	Lipid modifying agents	Ezetimibe	+		+							[253]
			Atorvastatin	+*									[254-256]
			Pitavastatin	+		+						+	[257]
			Pravastatin			+ (r)		+					[258, 259]
			Rosuvastatin	+		+						+	[260, 261]
Systemic hormons													
H	02A	Corticosteroids	Dexamethasone	+								-*	[100, 241]
Anti-infectives													
J	01A	Tetracyclines	Tetracycline	+				+					[258, 262]
	01C	Beta-lactams[a]		+		+		+				+ (r)	[258, 263-265]
	01F	Macrolides[a]		+	+	+ (r)							[266-268]
		Ketolides	Telithromycin	+		+ (r)							[269]
	01M	Fluoroquinolones[a]		+	+	+ (r)		+ (m)				+	[58, 268, 270-276]
	01X	Nitrofuranes	Nitrofurantoin									+	[277]
		Lipopeptides	Daptomycin	+									[40]
	02A	Azole antifungals	Itraconazole	+ (m)								-*	[164, 278]
	04A	Antimycobacterial antibiotics	Rifampicin	+ (m)									[279]
	05A	Reverse transcriptase inhibitors	Adefovir	- (CHO)	-			+	+		+	+ (m)	[44, 228, 280-283]
			Ganciclovir					+					[284]
			Zidovudine (AZT)					+				+	[282, 285]
		Protease inhibitors	Indinavir	+	+/-	+	-		-			-	[89, 162, 286-288]
			Lopinavir	+	-	+						- (m)	[289]
			Nelfinavir	+	+							-*	[89, 162, 288]
			Ritonavir	+	+/-	+	-		-			-*	[162, 286-288]
			Saquinavir	+	+/-	+	-		-			-*	[89, 162, 286-288, 290]
Antineoplastic and immunomodulating agents													
L	01B	Antimetabolites	Cladribine					+				+	[44, 291]
			Methotrexate	+	+	+	+	+	+			+[b] (and PG)	[292-299]
	01C	Plant alkaloids	Docetaxel	+		+					+		[300-302]
			Paclitaxel	+	-	+	-	-			+	-	[62, 228, 295, 300, 301, 303-305]
			Etoposide	+	+/-	+ (GC)	+ (and GC)	-		+	+	-	[62, 228, 281, 295, 304, 306-310]
			Vinblastine	+	+	+[#]		-			+		[228, 301, 304, 311-313]
			Vincristine	+	+[#]	+[#]	+/-	-			+		[228, 295, 301, 303, 307, 310, 314, 315]

(Table 2) Contd…..

ATC Code		Pharmacological Class	Drug	P-gp	MRP1	MRP2	MRP3	MRP4	MRP5	MRP6	MRP7	BCRP	Refs.
L	01D	Cytotoxic antibiotics	Actinomycin D	+	+								[304, 314]
			Daunorubicin	+	+ (GS)		-	-		+	+	+[b]	[62, 228, 281, 295, 303, 304, 308, 314, 316, 317]
			Doxorubicin	+	+ (GS)	+	-	-		+		+[b]	[62, 228, 295, 303, 304, 307, 308, 310, 316-318]
			Mitoxantrone	+	+[#]							+[b]	[62, 316, 319, 320]
	01X	Camptothecins	Irinotecan	+	+	+		+				+	[321-324]
			Topotecan	+				+				+	[325-328]
	-		Gimatecan	-		-		+				-	[329]
	-	Platinium compounds	Cisplatin			+[#]	-						[307, 310, 330]
	-	Protein kinase inhibitors	Imatinib	+								+/-*	[331-334]
			Lapatinib	+								+	[335]
	-	-	Becatecarin									+	[336]
	-	-	Flavopiridol	+ (m) /-	-							+/-	[62, 245, 337]
	04A	Immunosuppressants	Ciclosporin A	+*				-				-*	[100, 155, 324, 338]
			Tacrolimus	+								-*	[155, 338]
Musculo-skeletal system													
M	01A	Anti-inflammatory agents	Diclofenac	-		-						+	[142]
	04A	Antigout agents	Colchicine	+		+						-	[339]
Brain and nervous system													
N	02A	Opioid analgesics	Morphine	+ (CHO)		+ (GC)	+ (GC) (m)					-	[340-342]
			Oxycodone	+									[200]
	-	Analgesics	Asimadoline	+									[105]
	03A	Antiepileptics	Phenobarbital	+	-	-			-				[343, 344]
			Phenytoin	+	-	-			-			-	[343-345]
			Topiramate	+	-	-			-				[346]
	04B	Antiparkinsonian drugs	Bromocriptine	+ (m)									[347]
			Budipine	+ (m)									[348]
			L-dopa	+									[349]
	05A	Antipsychotic drugs	Fluphenazine	+									[350]
			Perazine	+									[350]
			Risperidone	+									[351]
	06A	Antidepressants	Citalopram	+ (m)									[352]
			Trimipramine	+ (m)									[352]
Antiparasitic products													
P	01B	Antiparasitics	Chloroquine	-	+								[353, 354]
			Mefloquine		+			+					[355]
			Quinine	+									[353]
	-		Quinacrine	+ (m)									[356]
	02C	Antihelmintics	Ivermectin	+									[100]
	-		Oxfendazole	-		-						+	[357]

(Table 2) Contd…..

ATC Code		Pharmacological Class	Drug	P-gp	MRP1	MRP2	MRP3	MRP4	MRP5	MRP6	MRP7	BCRP	Refs.
Respiratory system													
R	06A	Antihistaminics	Cetirizine	+	+								[358]
			Fexofenadine	+		+							[359, 360]
Various													
V	03A	Antidotes (morphinic antagonist)	Methadone	+								-	[341, 361]

Drug are classified according to ATC codes (Anatomical Therapeutic Chemical classification system; http://www.whocc.no/atc/).
All data refer to studies with human transporters, except when specifically indicated: (m) mouse; (r) rat; (CHO) Chinese hamster ovary cells.
Key: +, substrate; -, non substrate; *, modulator/inhibitor [143]; #, transport is dependent upon the presence of glutathione; GS, glutathione conjugate; GC, glucuronide conjugate; PG, polyglutamate conjugate.
[a], does not apply to the whole class (some members are substrates, others, not); [b], BCRP substrate specificity is affected by mutations at amino acid 482 [62].

Table **2** summarizes our current knowledge on the active transport of drugs by the main MDR ABC transporters. A first observation is that a single transporter can affect a very large number of molecules, belonging to a wide variety of pharmacological classes and presenting markedly remote chemical structures. P-gp substrates are mostly organic amphipathic molecules, ranging in size from less than 200 Da to almost 1900 Da. Most of them are neutral or basic compounds, but zwitterionic and negatively charged compounds (like methotrexate) can also be transported. Among MRPs, MRP4 and MRP5 have the particularity to transport cyclic nucleotides and purine analogues [44-46], but not anthracyclines, taxanes, or vinca alkaloids. BCRP shows a broad substrate specificity, with partial overlap with P-gp substrates. On the other hand, all drugs belonging to a same pharmacological class are not necessarily substrates for the same transporter. All together, these data suggest that recognition by MDR transporters depends on molecular determinants that have nothing in common with those defining the high specificity of drug-target interaction in most pharmacological models (classical model of the key-and-lock recognition [47]). Yet, converging evidence from experimental studies and molecular modeling tend to indicate that these are the global physico-chemical properties of the molecule rather than the presence of specific substituents that drive substrate recognition. Tentative 'pharmacophores' have been progressively built up that allow to predict possible interactions, mainly with P-glycoprotein, and are now used for *in silico* screening [48, 49]. The features identified include the presence of hydrogen bond acceptor, hydrophobic and aromatic areas, and positive ionizable group at appropriate distance from one another [50]. Another factor that can contribute to broad substrate specificity is the fact that MDR transporters possess several binding sites in the transmembrane domains, as demonstrated for P-gp [51-53], MRP2 [54] or BCRP [55], which can probably accommodate different substrates [56].

A second observation is that a single molecule can be substrate for different transporters. At the molecular level, this indicates that common features may dictate recognition by different transporters. In this respect, it is interesting to note that this may even apply to totally unrelated transporters, as those conferring resistance to antibiotics in bacteria. For example, ciprofloxacin but not moxifloxacin, is substrate of murine Mrp4 [57, 58] as well as of efflux pumps conferring resistance to fluoroquinolones in *Staphylococcus aureus*, *Streptococcus pneumoniae,* or *Listeria monocytogenes* [59-61]. At the physiological level, this redundancy between transporters may compensate for the poor expression of a given transporter in a particular tissue and/or for alteration of activity in mutated proteins. Mutagenesis studies have indeed shown that substrate specificity can be affected by a single amino acid change (see for example [62] for BCRP or [63] for P-glycoprotein). Indeed, *in vivo* also, variations in ABC transporters expression between individuals is well documented [64, 65], as well as genetic polymorphisms (see for review [66] for P-gp and MRP2 and [67] for BCRP). These polymorphisms might however be clinically relevant only at certain drug doses.

A third observation is that P-glycoprotein seems by far to be the broadest spectrum transporter. This conclusion needs however to be taken with caution, as P-glycoprotein is also the most widely studied transporter. Empty cells in Table **2** need thus to be interpreted as an absence of data and not necessarily as an absence of transport. Other possible limitations of the data presented in this Table are that some of them have been performed in animal cells (exploring therefore transport capacity of the animal transporter), or in animal cells transfected with human transporter (but with the remaining background of the other transporters expressed by the animal cell) or using knockout animals. Transposition of the results to human needs therefore careful appreciation due to interspecies substrate discrepancies. Thus, whereas mouse Bcrp1 was functionally comparable with human BCRP in a murine fibroblast cell line [68], interspecies differences do exist between Bcrp1/BCRP in hepatocytes [69], as well as between murine and human MRP2/Mrp2 [70, 71], or P-gp [72, 73].

Consequences for Drug Absorption (Intestinal Barrier)

Drugs administrated by oral route must pass through several barriers before reaching their target site, the first one being the intestinal epithelium. Due to their high expression in the small intestine and to their co-localization at the apical membrane of enterocytes, P-gp, MRP2, and BCRP play a key role in limiting the absorption of drugs by expelling them back to the intestinal lumen [74, 75]. Expression of transporters along the small intestine is not uniform and regional differences have been reported (see for review [75]): whereas P-gp expression is higher in the ileum [76], MRP2 and BCRP expression are higher in jejunum [77, 78]. This will affect locally drug absorption at the intestinal

barrier. For example, a significant inverse correlation was found between ciclosporin A absorption and intestinal P-gp mRNA levels along the gastrointestinal tract [79].

To date, the role of P-gp is the most documented [80]. In the mice, however, Bcrp1 has been shown to limit the oral bioavailability of the anticancer drug topotecan [81], and to protect the animals against ingested dietary carcinogens (such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, PhIP) [82] or phototoxins like pheophorbide A [83]. On the contrary, MRP transporters expressed at the basolateral side of the cells may increase drug absorption. This has been demonstrated for ampicillin [84] or adefovir [85] using *in vitro* models of intestinal barrier.

The tools developed to study the role of P-gp in intestinal drug absorption consist of *in vitro* models of Caco-2 cell monolayers [86] and *in vivo* models with knockout mice [87]. Mice express two isoforms of P-gp, namely Mdr1a and Mdr1b, which both act as multidrug transporters; however, Mdr1b is not detected in the intestine. *Mdr1a* (-/-) mice allowed for example to demonstrate the major role of P-gp in the pharmacokinetics of paclitaxel [88] or HIV protease inhibitors (indinavir, nelfinavir and saquinavir [89]), since drug plasmatic concentrations were significantly higher in *Mdr1a* (-/-) mice than in WT mice (6-fold higher for paclitaxel, and 2- to 5-fold higher for HIV protease inhibitors). Studies with healthy volunteers allowed to confirm the importance of P-gp expression levels [79] or of the co-administration of pump inhibitors for drug absorption [90].

Moreover, detoxifying enzymes of cytochrome P450 family are likely to act in synergy with ABC transporters to decrease drug absorption [91, 92]. Cytochrome P450 3A4 (CYP3A4) accounts for nearly 70% of all CYP enzymes expressed in small intestine [93]. It displays a substantial overlap in substrate specificity and colocalizes with P-gp in enterocytes [94]. Recently developed models of *Mdr1a/1b* (-/-), *Cyp3a* (-/-), and *Cyp3a/Mdr1a/1b* (-/-) mice will thus be of prime interest to evaluate the respective importance of metabolism and efflux in drug disposition. Of high interest, recent data obtained with this model suggest that there is a high degree of synergy between Cyp3a and Mdr1a. For example, a >70-fold increase in systemic exposure to docetaxel is observed after oral administration to *Cyp3a/Mdr1a/1b* (-/-) mice *vs.* a 12-fold increase in *Cyp3a* (-/-) mice and a 3-fold increase in *Mdr1a/1b* (-/-) mice [95]. Mathematical models have been developed to predict the change in AUC mediated by each of these systems for drugs that are common substrates [96]. Yet, the observation of synergistic effects makes probably largely pointless evaluations of the individual contribution of each of these mechanisms with respect to modifications of drug bioavailability *in vivo.*

Consequences for Drug Distribution

ABC transporters located at the blood-brain barrier (BBB), the blood-CSF barrier, the blood-placental barrier, or the blood-testis barrier restrict the penetration of xenobiotics into the central nervous system, the foetus (via the placenta) or the testis. While this contributes to protect these vulnerable territories, it also compromises drug accessibility in pathological situations. This is most conspicuously the case for central nervous diseases (neurodegenerative diseases, intracranial tumors, dementia, epilepsy, meningitis…). Two physiological barriers separate the brain from the bloodstream. The blood-brain barrier (BBB) is made of endothelial cells of the brain microvasculature that isolate the cerebral blood from the brain interstitial fluid. Tight junctions between these cells limit the paracellular flux of hydrophilic molecules across the BBB, so that only lipophilic molecules with low molecular weight can passively diffuse. The blood-cerebrospinal fluid (CSF) barrier is formed by a single layer of choroid plexus epithelial cells that separates the plexus blood from the CSF. BCRP and P-gp are the main ABC transporters expressed at the human BBB [97]; they are both localized at the apical (or luminal) pole of the BBB where they transport drugs from the brain to the blood. MRPs are also detected but with a lower expression; their functional role at the BBB still needs to be clearly determined [98].

The first studies investigating the influence of ABC transporters at the BBB were performed *in vitro*, using cultures of brain endothelial cells. These cells however do not always exhibit all the properties of *in situ* brain microvessel endothelial cells [98]. P-gp-knockout mice models were thereafter used to demonstrate the implication of P-gp to limit drugs entry into the brain, Mdr1a being the major P-gp isoform present at the BBB. The first studies with *Mdr1a* (-/-) mice showed that they were almost 100-fold more sensitive to the neurotoxic effects of ivermectin, an antiparasitic compound [87] than wild-type mice. Many other P-gp substrates, such as digoxin [99, 100], ciclosporin A [100], loperamide, domperidone and ondansetron [101], HIV protease inhibitors (indinavir, saquinavir, nelfinavir) [89], or paclitaxel [102] are accumulated in the brains of P-gp-deficient mice up to 35- or 40-fold higher than in WT mice, clearly documenting the role of P-gp as a gatekeeper at the luminal side of the BBB [103]. Several studies also evidenced a more marked implication of Mdr1a at the BBB than at the intestinal barrier by comparing the increase in drug concentration in the brain *vs*. the intestine of *Mdr1a* (-/-) or *Mdr1a/1b* (-/-) mice as compared to wild-type animals (4.4- to 9.6-fold *vs*. 2-fold for vinblastine [104]; 9-fold *vs*. no effect for asimadoline, an experimental analgesic [105]). More recently, positron emission tomography (PET) and single photon emission computed tomography (SPECT) imaging techniques [106] with radiolabeled efflux pump substrates have allowed non-invasive studies in animals and humans and a direct visualization of drug transporter function at the BBB [107, 108].

Although expressed at the BBB [109], Bcrp1 seems to have a moderate role in the transport of substances known to be BCRP substrates, such as imatinib [110] and mitoxantrone [111], or of xenobiotics that are also P-gp substrates [112, 113]. Yet, other studies showed the Bcrp1 acts synergistically with P-gp to limit the brain penetration of topotecan [114] and lapatinib [115]. In case of P-gp deficiency, however, Bcrp1 expression at the BBB increases, which is accompanied by greater export of its substrates, like mitoxantrone or prazosin [116]. Mrp4 presents the particularity of being expressed at the apical membrane of endothelial cells at the BBB but at the basolateral membrane of epithelial cells at the blood-CSF barrier. This dual localization allows for clearance of Mrp4 substrates from both the CSF

and the brain, as shown for topotecan [117]. However, this effect has been observed in rodents and might not be relevant in humans, where MRP4 expression seems to be very low [118].

In the placenta, P-gp expressed in trophoblasts protects the fetus from potential teratogenic compounds [119], and from many drugs like digoxin, saquinavir or paclitaxel [120] extruding them into the maternal blood. Likewise, Bcrp1, expressed in placental syncytiotrophoblasts [109], limits the foetal penetration of topotecan [81]. Again the role of active transporters as a limitation to the permeability of the foeto-maternal barrier may rationalize clinical observations, for example the lack of efficacy of protease inhibitors for preventing HIV transmission in pregnant women [121].

Consequences for Drug Elimination

A role for intestinal P-gp in the elimination of drugs from the blood to the gut lumen has been described [122], but the main routes of drug elimination remain through biliary excretion and renal clearance.

Biliary Excretion of Drugs

In the liver, a lot of transporters are involved not only in the excretion of bile constituents, but also of xenobiotics and metabolites produced by Phase I and Phase II enzymes. These include MDR transporters, but also other ABC transporters like BSEP (Bile Salt Export Pump, ABCB11) and Solute-Linked Carrier transporters [123, 124]. P-gp, MRP2 and BCRP are localized at the canicular membrane of hepatocytes and secrete metabolized xenobiotics into the bile. MRP1, MRP3 and MRP4 are expressed at the basolateral membrane and extrude metabolites in the blood, from where they can be eliminated by the kidneys (for a review, see [125]). Hepatic cells appear thus as a hub, orientating the route of elimination of metabolized drugs depending on their affinity for apical or basolateral transporters.

Transporters can also cooperate at the level of different barriers to efficiently reduce drug concentrations in the blood. For example, a complementary role of P-gp and Mrps has been evidenced for paclitaxel [126] and etoposide [127]. While P-gp is mainly involved in restricting their intestinal absorption, Mrp2 dominates in their hepatobiliary excretion. Moreover, in Mrp2 deficient animals (Mrp2 knockout mice), Mrp3 can secrete etoposide metabolites from the liver to the blood, from where they are further eliminated in urine [127]. Thus, MRP3 is considered to function as a backup detoxifying pathway for hepatocytes, since its expression is increased when the normal canicular route is damaged by cholestatic diseases or when the function of MRP2 is impaired [128].

Renal Drug Excretion

In renal epithelial cells, P-gp, MRP2, and MRP4 are expressed at the apical (luminal) membrane, whereas MRP1 and MRP6 are localized on the basolateral membrane [129]. Moreover, P-gp, MRP2, MRP4, and MRP6 are expressed in renal proximal tubules, whereas MRP1 is localized in distal tubules and collecting ducts [129], protecting distal part of the nephron from toxic drug accumulation which may occur with water reabsorption. BCRP protein expression in kidney has been recently evidenced, with also a localization in proximal tubules [23] but its role in renal drug efflux remains to be clearly determined.

Beside their role in drug elimination, MDR transporters may also exert a protective role on the kidneys themselves, as these organs are particularly exposed to toxic compounds. In patients (or animals) with chronic renal failure, it has been observed that the renal expression of P-gp [130] or of Mrp2 [131] is increased while that of uptake transporters is decreased. This may help the sick organ to eliminate toxins. Modifications of the expression of MDR transporters may also contribute to modulate drug nephrotoxicity. It has been shown for example that the expression level of P-gp is lower in kidney graft recipients treated with ciclosporin A than in those treated with tacrolimus. This is correlated with a longer graft survival in the tacrolimus patients, attributed to a higher nephrotoxicity in the ciclosporin A group [132]. Overexpression of several MDR transporters (P-gp, Mrp2, Mrp4, Mrp5 [133]) and down regulation of influx transporters (OAT and OCT) has also been evidenced in mice treated with cisplatin, another nephrotoxic drug, even if its transport is not documented for all of them (see Table **2**).

MDR EFFLUX PUMPS AND TRANSPORTER-MEDIATED DRUG-DRUG INTERACTIONS

Polymedication is very frequent in clinical practice, especially in the elderly. It is often the cause of iatrogenic adverse reactions related either to drug-drug interactions or to inappropriate dosing due to organ insufficiency in old patients. Some mechanisms of pharmacokinetic interactions are now quite well characterized, like those mediated by the administration of inhibitors or inducers of cytochromes P450 or the formation of complexes between cationic and anionic compounds. Yet, it now appears that MDR transporters can also play a major role in drug –drug interactions. The most popular example is probably that of flavonoids present in grapefruit juice, which can inhibit both the P-gp-mediated efflux and the CYP3A4-mediated metabolism of many drugs in enterocytes, improving thereby their bioavailability [134, 135]. There is considerable overlap between CYP3A4 and P-glycoprotein substrates [136], so that both systems will often be involved in drug interactions, resulting in complex pharmacokinetic profiles of multidrug regimens [137]. As compared to CYP-mediated drug interactions, those mediated by MDR transporters have however the particularity of possibly affecting drug concentration in a specific body compartment (such as the brain) without modifying blood levels.

Drug-drug interactions related to MDR transporters can occur by two main mechanisms. The first one is a competition between drugs (substrates or modulators of the pump) for the binding site(s) of the transporter, which can impair the transport of one or the two interacting drugs. The second is a change in the expression level of the MDR transporter upon exposure to a given drug, but which can affect the transport of any other drug substrate of the same pump. These interactions are not always deleterious, one drug being able to boost the absorption of the second one. This is well exemplified in Kaletra®, which consists of the combination of a therapeutic dose of lopinavir and a low

dose of ritonavir, which only serve for inhibiting lopinavir efflux and metabolism, hence increasing its bioavailability [138-140].

Competition for Drug Binding Site

A combination of an efflux pump substrate with a well-characterized inhibitor/modulator can be useful to increase intestinal absorption or penetration into specific tissues, but it can also lead to adverse effects by decreasing drug elimination. On the other hand, the co-administration of two drugs substrates for the same transporter may sometimes result in unexpected and/or unwanted effects. One may anticipate that the drug with the highest affinity will be more efficiently transported, and thus inhibit the transport of the other drug. Yet, if the mechanism of the interaction is competitive, the concentration ratio between the two drugs may also play a critical role in determining which one will influence the transporter of the other one. Moreover, other mechanisms of interaction than simple competition for transport have been described, for example, allosteric modification by binding to a modulator site (see for example diclofenac, which inhibits the transport of anionic substrates by MRP2 [141] but stimulates that of amphiphilic substrates [142]). On these bases, it is clear that transporter-mediated drug interactions are not easy to predict *in vivo*, and are often understood *a posteriori.* Methods to accurately predict such interactions are therefore needed [96].

A series of drugs, which were first documented as being P-gp substrates, are now widely used both *in vitro* and *in vivo* for their modulator activity (among others, quinine and quinidine, verapamil, ciclosporin A and nifedipine; they constitute the first generation of P-gp modulators [143, 144]). Using P-gp knockout mice, Fromm *et al.* [145] showed that co-administration of quinidine increases digoxin concentrations in plasma and brain (by 73.0% and 73.2%, respectively) of wild-type mice, but not in *Mdr1a* (-/-) mice, demonstrating that quinidine is not only a substrate, but also a potent inhibitor of P-gp. In accordance with these results, a study with human volunteers showed that digoxin intestinal absorption increased from 22.3 ± 8.9% to 55.8 ± 21.2% of the dose when co-administrated with quinidine [90]. Digoxin oral bioavailability is also increased when co-administrated with talinolol [146], with a 23% increase of the area under the concentration-time curve AUC(0-72h), or clarithromycin [147] (1.7-fold increase in AUC(0-24h)), whereas its renal elimination is reduced when co-administrated with verapamil [148]. In another study with healthy male volunteers, quinidine caused an increase of loperamide transport into the brain, leading to several side effects, although the blood plasma concentration of loperamide remained unchanged [149].

Ciclosporin A, another well-known P-gp substrate [100], is also able to act as an inhibitor, increasing taxane (paclitaxel or docetaxel) oral bioavailability in wild-type mice [150] (from 9.3% up to 67% when co-administrated with ciclosporin A) as well as in cancer patients [151, 152] (from 4-8% for taxane alone, up to 47% or 88%, depending on the taxane, in presence of ciclosporin A). Similarly, the increased bioavailability and reduced clearance of the BCRP substrate irinotecan in patients treated concomitantly with ciclosporin A [153, 154] has been attributed to the inhibition of BCRP by ciclosporin A [155]. The clinical efficacy of ciclosporin A as a pump modulator is thus related to its ability to inhibit different MDR transporters (P-gp, BCRP, MRP1 [156]).

Anti-HIV therapy requires the combination of three or four antiretroviral drugs from different classes. Many anti-HIV drugs have been demonstrated as being substrates for MDR transporters, mainly P-gp and MRP2 (see Table **2**). However, ritonavir also behaves as a P-gp inhibitor and decreases digoxin clearance by 35%, in humans, likely because both drugs compete with P-gp for renal elimination [157]. P-gp and CYP3A4 inhibition by ritonavir or other protease inhibitors has also been evoked to explain the increased blood concentrations of tacrolimus [158], fexofenadine [159] or loperamide [160]. This could apply to much more classes of drugs that are substrates of both P-gp and CYP 3A4 [161]. Moreover, protease inhibitors are also inhibitors (but not substrates) of BCRP [162, 163], and could therefore also affect the pharmacokinetic profile of drugs that are substrates of this transporter. The same reasoning could apply to antifungal agents, which are substrates of P-gp but inhibitors of BCRP [164].

Several drug-drug interactions have been reported with the antifolate drug methotrexate. Co-administration of benzimidazole proton-pump inhibitors significantly inhibits BCRP-mediated transport of methotrexate *in vitro*, and pantoprazole reduces its clearance *in vivo* in mice (1.9-fold), possibly via competition for BCRP [165]. Co-administration of nonsteroidal anti-inflammatory drugs (NSAIDs) [166] also modifies methotrexate pharmacokinetics, possibly by inhibiting its renal tubular secretion via MRP2 and MRP4 [141, 167]; *in vitro* diclofenac inhibits BCRP-mediated methotrexate transport [142].

Much more interactions have been described in cellular or *in vitro* models. For example, bromocriptin increases L-dopa cellular accumulation about 2.05-fold in a rat brain endothelial cell model by inhibiting P-gp [168], whereas amiodarone inhibits digoxin secretion through P-gp in kidney epithelial cells [169]. Interactions with anticancer drugs have also been demonstrated. The antibiotics ofloxacin and erythromycin enhance vincristine accumulation in MRP1-overexpressing cells [170], and opiates (methadone and morphine) inhibit paclitaxel uptake by P-gp in human placental inside-out vesicles [171]. On the contrary, transport of paclitaxel, docetaxel, and saquinavir in MDCK cells overexpressing MRP2 is stimulated by diclofenac [142]. Further investigations are needed however to determine whether these are relevant in the clinics, as concentrations used *in vitro* are often supratherapeutic.

Drug-induced Change in Expression of MDR ABC Transporters

Regulation of transporters expression has been mainly studied in the liver, a key organ for drug detoxification and disposition (for comprehensive reviews, see [172, 173]). Several nuclear receptors like the pregnane X receptor (PXR, also referred as the steroid and xenobiotic receptor SXR), constitutive androstane receptor (CAR), peroxisome proliferator activated receptor alpha (PPARα), or nuclear factor-E2-related factor (Nrf2) are implicated in the induction by

xenobiotics of ABC transporters (P-gp, MRP2 [174], MRP3 [175], Mrp4 [176] or BCRP [177]), as well as of cytochromes P450 [178] or of uptake transporters (OATP) [179], enabling a coordinated response to drug injury. Nuclear receptors regulate target gene transcription in a ligand-dependent manner. Ligand binding promotes their activation and translocation to the nucleus, where they form homo- or heterodimers that bind to specific response elements within regulatory regions of the target gene. Several drugs are able to bind to and activate nuclear receptors, such as rifampicin, clotrimazole, phenobarbital, dexamethasone, nifedipine, or midazolam [180], and therefore to modulate MDR transporter expression (see Table **3**) [178, 181]. *In vitro*, other drugs induce rather gene amplification [182].

Rifampicin is known for a long time as an inducer of P-gp and MRP2 [183, 184], through a PXR-activation mechanism [185]. In human healthy volunteers, rifampicin treatment increases intestinal P-gp level, thus affecting oral bioavailability of several drugs, such as digoxin [184], talinolol [186], fexofenadine [187] or ciclosporin A [188]. Mice expressing human PXR and treated with rifampicin were also much less susceptible to methadone antinociceptive effect, demonstrating the increase of P-gp activity at the BBB after rifampicin treatment [189].

HIV protease inhibitors like amprenavir and nelfinavir [190], ritonavir [191, 192] or atazanavir [193] can induce intestinal P-gp overexpression in animals and in cultured cells [194, 195], through binding and activation of PXR, at clinically-relevant concentrations for ritonavir [196]. Ritonavir also induces MRP1 overexpression *in vitro* [191]. However, patients treated with protease inhibitors do not exhibit an increase in P-gp expression in lymphocytes, as compared to patients treated with other classes of antiretrovirals [197]. Yet, non-nucleoside and nucleoside reverse transcriptase inhibitors also induce intestinal P-gp expression *in vitro* probably via a PXR pathway [198, 199], making the previous study difficult to interpret.

In rats, repeated administration of oxycodone (an opioid agonist used for the management of pain in cancer patients) causes P-gp overexpression (in liver, kidney, and brain), and affects tissue concentration of paclitaxel [200]. Celecoxib, a NSAID, induces an increase in MRP4 and MRP5 expression *in vitro* at clinically relevant concentrations [201]. This could explain the lack of improvement in response rate observed in clinical trials examining celecoxib combined with irinotecan for solid malignancies [202]. Carbamazepine, an antiepileptic drug known as a CYP3A4 inducer, has been shown to induce both intestinal P-gp and MRP2 in human healthy volunteers, which affects talinolol pharmacokinetics [203]. Other antiepileptic drugs, among which phenobarbital (a known PXR activator), also increase P-gp, MRP1 and MRP2 expression levels after long-term exposure of rat brain microvascular endothelial cells [204, 205] as well as in rat brain [206]. This effect is associated with an activation of PXR and CAR receptors [205].

Acquired MDR phenotype in cancer cells often results from the overexpression of ABC transporters able to expel anticancer drugs out from the cells [10, 42]. This suggests that anticancer drugs can induce the expression of the corresponding transporter. Thus, resistant cell lines obtained *in vitro* after chronic exposure to various anticancer agents (see Table **3**) do indeed overexpress ABC transporters. The same strategy could be applied to other drug substrates, provided they can exert a certain toxicity on cells allowing to select those having acquired resistance. Successful examples include mouse macrophages exposed to ciprofloxacin, which overexpress Mrp4 [58, 207] or human erythroleukemia cells exposed to adefovir, which overexpress an indomethacin-sensitive efflux pump (later identified as being also MRP4) [208]. This strategy is thus very useful to obtain cells overexpressing efflux pumps as tools for molecular studies and characterization of drug transport [209]. The conditions needed to select cells *in vitro* are not relevant from the clinical situation (high concentrations; prolonged exposure), but clinical data suggests this also occurs during therapy. Induction of P-gp expression during treatment has been demonstrated for example in patients treated for bladder cancer with doxorubicin [210]. Overexpression of P-gp, MRPs or BCRP at the surface of cancer cells is frequently reported in tumors and constitute a poor prognosis factor [211, 212]. Interestingly also, these transporters show higher expression levels at the BBB in drug refractory epilepsy [213, 214].

MDR ABC TRANSPORTERS AS A DRUG TARGET

Considerable effort has been made over the last decade to develop efflux pump inhibitors as a way to improve efficacy of anticancer agents (see for recent reviews [215 and 216]). Yet, if *in vitro* or animal data are promising, success is limited in clinical trials, probably in relation with the pleiotropic character of the MDR transporters and with the difficulty of inhibiting transporters that have physiological roles without causing toxicity.

In a more general context, inhibition of apical transporters like P-gp and/or BCRP is also an attractive strategy to improve oral bioavailability and CNS penetration of drug substrates [103, 110, 120] but it may face the same limitations.

Another strategy could therefore rather consist of trying to select drugs that are poor substrates for efflux transporters. High throughput methods of *in vitro* and *in silico* screening should be helpful in this respect.

CONCLUSION

There is no doubt that active efflux transport should now be considered as a part of the evaluation of the pharmacokinetic profile of a drug, to the same extent as its metabolism by hepatic enzymes. Variations in the expression profile of transporters should also be considered with care to explain inter-individual variability.

The importance of characterizing transport by MDR efflux pumps is now recognized also by health authorities. In its last drug interaction guidance, the US Food and Drug Administration recommends indeed to test for transport, inhibition or induction of P-glycoprotein by new drugs, as a way to predict potential drug-drug interactions [217-219].

Table 3. Drug Inducers of MDR ABC Transporters Expression

ATC code		Pharmacological class	Drug	P-gp	MRP1	MRP2	MRP3	MRP4	MRP5	BCRP	Refs.
Cardiovascular system											
C	01A	Cardiac glycosides	Digoxin	+ (l)							[362]
	01B	Antiarrhythmics	Amiodarone	+ (s)							[181]
			Quinidine	+ (s)							[363]
	02A	Anti-hypertensive agents	Reserpine	+ (s)							[181]
	08C	Calcium channel blockers (vascular)	Nifedipine	+ (s)			+ (s)				[175, 181]
	08D	Calcium channel blockers (cardiac)	Verapamil	+ (s)							[181]
	10A	Lipid modifying agents	Atorvastatin	+ (s)							[363]
Genito-urinary system and sex hormones											
G	01A	Antiinfectives (triazole antifungal)	Clotrimazole	+ (s)			+ (s)				[175, 181]
Systemic hormons											
H	02A	Corticosteroids	Dexamethasone	**+** (r,s)		**+** (r,s) / - (s)	- (s)		- (s)	+ (r,s)	[364-366]
Anti-infectives											
J	01C	Beta-lactams	Flucloxacillin	+ (s)							[367]
	01F	Macrolides	Erythromycin	+ (s)							[181]
	01G	Aminoglycosides	Gentamicin			**+** (r,l)					[368]
	01M	Fluoroquinolones	Ciprofloxacin			+ (m,l)		+ (m,l)			[58]
	04A	Antimycobacterial antibiotics	Rifampicin	**+** (s,l)	+ (p,l) / - (s)	+ (s,l)	+ (s)		+ (s)	+ (s)	[175, 183-185, 366, 369-371]
	05A	Reverse transcriptase inhibitors	Zidovudine (AZT)	+ (l)				+ (l)	+ (l)		[41]
		Protease inhibitors	Atazanavir	+ (s)							[193]
			Nelfinavir	**+** (r,l)							[190]
			Ritonavir	+ (s), **+** (r,s)	+ (s)						[191, 192]
		Non-nucleoside reverse transcriptase inhibitors	Delavirdine	+ (s)							[198]
		-	Neviradine	+ (s)							[199]
Antineoplastic and immunomodulating agents											
L	01C	Plant alkaloids	Docetaxel	+ (s)							[372]
			Paclitaxel	+ (s)							[372]
			Vinblastine	+ (s)	+ (s)	- (s)					[366, 372]
			Vincristine	+ (s)		+ (s)	+ (s)				[369, 372]
	01D	Cytotoxic antibiotics	Doxorubicin	+ (s), **+** (l)	- (s)	- (s)			+ (s)	+ (m,l)	[68, 210, 366, 373, 374]
			Mitoxantrone	+ (s)						+ (l)	[182, 375, 376]
	01X	Camptothecins	Topotecan	+ (s)						+ (l)	[328, 363]
		Platinium compounds	Cisplatin	+ (p,s)	- (s)	+ (s)	+ (s)		**+** (s,l)		[366, 377-379]
		Protein kinase inhibitors	Imatinib	+ (l)	- (l)	- (l)				+ (l)	[380]
	02B	Hormon antagonists	Tamoxifen	+ (s)	- (s)	**+** (mo,l) / - (s)	- (s)		- (s)		[366, 372, 381]
	04A	Immunosuppressants	Sirolimus	+ (s)							[181]
			Tacrolimus	+ (s)							[181]

(Table 3) Contd .....

ATC code		Pharmacological class	Drug	P-gp	MRP1	MRP2	MRP3	MRP4	MRP5	BCRP	Refs.
Musculo-skeletal system											
M	01A	Anti-inflammatory agents	Celecoxib		- (s)	- (s)		+ (s)	+ (s)		[201]
Brain and nervous system											
N	02A	Opioid analgesics	Morphine	**+** (r,l)	**+** (r,l)					**+** (r,l)	[382, 383]
			Oxycodone	**+** (r,l)							[200]
	03A	Antiepileptics	Carbamazepine	**+** (l)		**+** (l)					[203]
			Phenobarbital	+ (s)	-	+ (s)				+ (s)	[174, 181, 366, 370]
	04B	Antiparkinsonian drugs	Bromocriptine	+ (m, s)							[384]
	05C	Hypnotics and sedatives	Midazolam	+ (s)							[181]
Antiparasitic products and insecticides											
P	01B	Antimalarials	Artemisinin	+ (s)							[385]

Drugs are classified according to ATC codes (Anatomical Therapeutic Chemical classification system; http://www.whocc.no/atc/).
Induction has usually been demonstrated *in vitro* (at mRNA and/or protein levels); symbols in bold correspond to *in vivo* induction. Studies were performed in animals: m, mouse; r, rat; p, pig; mo, rhesus monkeys; or in humans/human cell lines (no indication). Induction has been performed for short time (s) (≤ 72h), or long time (l) (> 3 days) periods.

Appropriate models are therefore critically needed to evaluate drug transport by specific efflux pumps. P-gp role is now appropriately evaluated, using reliable *in vitro* and *in vivo* procedures. Interactions caused by other MDR transporters still need to be examined on a case-by-case basis, as standard procedures are lacking. Furthermore, we also need filling the gap between *in vitro* and *in vivo* data to accurately predict the role of MDR efflux pumps in drug transport and drug interactions.

ACKNOWLEDGEMENTS

B.M. was post-doctoral fellow of the program FIRST post-doc of the *Region Wallonne* and F.V.B. is Maître de Recherches of the Belgian *Fonds de la Recherche Scientifique (FNRS-FRS)*.

REFERENCES

[1] Dean M. The genetics of ATP-binding cassette transporters. Methods Enzymol 2005; 400: 409-29.
[2] Busch W, Saier MH Jr. The IUBMB-endorsed transporter classification system. Mol Biotechnol 2004; 27: 253-62.
[3] Saier MH Jr., Tran CV, Barabote RD. TCDB: the Transporter Classification Database for membrane transport protein analyses and information. Nucleic Acids Res 2006; 34: D181-6.
[4] Saier MH Jr., Yen MR, Noto K, Tamang DG, Elkan C. The Transporter Classification Database: recent advances. Nucleic Acids Res 2009; 37: D274-8.
[5] Vasiliou V, Vasiliou K, Nebert DW. Human ATP-binding cassette (ABC) transporter family. Hum Genom 2009; 3: 281-90.
[6] Kindla J, Fromm MF, Konig J. *In vitro* evidence for the role of OATP and OCT uptake transporters in drug-drug interactions. Expert Opin Drug Metab Toxicol 2009; 5: 489-500.
[7] Kalliokoski A, Niemi M. Impact of OATP transporters on pharmacokinetics. Br J Pharmacol 2009; 158: 693-705.
[8] Chapuy B, Koch R, Radunski U, *et al.* Intracellular ABC transporter A3 confers multidrug resistance in leukemia cells by lysosomal drug sequestration. Leukemia 2008; 22: 1576-86.
[9] Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2002; 2: 48-58.
[10] Eckford PD, Sharom FJ. ABC efflux pump-based resistance to chemotherapy drugs. Chem Rev 2009; 109: 2989-3011.
[11] Thiebaut F, Tsuruo T, Hamada H, *et al.* Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proc Natl Acad Sci USA 1987; 84: 7735-8.
[12] Colabufo NA, Berardi F, Contino M, Niso M, Perrone R. ABC pumps and their role in active drug transport. Curr Top Med Chem 2009; 9: 119-29.
[13] Szakacs G, Varadi A, Ozvegy-Laczka C, Sarkadi B. The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). Drug Discov Today 2008; 13: 379-93.
[14] Scherrmann JM. Transporters in absorption, distribution, and elimination. Chem Biodivers 2009; 6: 1933-42.
[15] Aller SG, Yu J, Ward A, *et al.* Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 2009; 323: 1718-22.
[16] Zolnerciks JK, Wooding C, Linton KJ. Evidence for a Sav1866-like architecture for the human multidrug transporter P-glycoprotein. FASEB J 2007; 21: 3937-48.
[17] DeGorter MK, Conseil G, Deeley RG, Campbell RL, Cole SP. Molecular modeling of the human multidrug resistance protein 1 (MRP1/ABCC1). Biochem Biophys Res Commun 2008; 365: 29-34.
[18] Ravna AW, Sager G. Molecular model of the outward facing state of the human multidrug resistance protein 4 (MRP4/ABCC4). Bioorg Med Chem Lett 2008; 18: 3481-3.
[19] Becker JP, Depret G, Van Bambeke F, Tulkens PM, Prevost M. Molecular models of human P-glycoprotein in two different catalytic states. BMC Struct Biol 2009; 9: 3.
[20] Stockner T, de Vries SJ, Bonvin AM, Ecker GF, Chiba P. Data-driven homology modelling of P-glycoprotein in the ATP-bound state indicates flexibility of the transmembrane domains. FEBS J 2009; 276: 964-72.
[21] Ravna AW, Sager G. Molecular modeling studies of ABC transporters involved in multidrug resistance. Mini Rev Med Chem 2009; 9: 186-93.
[22] Xing L, Hu Y, Lai Y. Advancement of structure-activity relationship of multidrug resistance-associated protein 2 interactions. AAPS J 2009; 11: 406-13.
[23] Huls M, Brown CD, Windass AS, *et al.* The breast cancer resistance protein transporter ABCG2 is expressed in the human kidney proximal tubule apical membrane. Kidney Int 2008; 73: 220-5.
[24] Stojic J, Stohr H, Weber BH. Three novel ABCC5 splice variants in human retina and their role as regulators of ABCC5 gene expression. BMC Mol Biol 2007; 8: 42.
[25] Lamba JK, Adachi M, Sun D, *et al.* Nonsense mediated decay downregulates conserved alternatively spliced ABCC4 transcripts bearing nonsense codons. Hum Mol Genet 2003; 12: 99-109.

[26] Kao HH, Chang MS, Cheng JF, Huang JD. Genomic structure, gene expression, and promoter analysis of human multidrug resistance-associated protein 7. J Biomed Sci 2003; 10: 98-110.
[27] Nies AT, Keppler D. The apical conjugate efflux pump ABCC2 (MRP2). Pflugers Arch 2007; 453: 643-59.
[28] Back M. Leukotrienes: potential therapeutic targets in cardiovascular diseases. Bull Acad Natl Med 2006; 190: 1511-8.
[29] Giannitsas K, Mitropoulos D, Konstantinopoulos A, Athanasopoulos A, Perimenis P. Phosphodiesterase-5 inhibitors in the treatment of lower urinary tract symptoms and benign prostatic hyperplasia. Expert Opin Pharmacother 2008; 9: 1687-93.
[30] Deeley RG, Westlake C, Cole SP. Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiol Rev 2006; 86: 849-99.
[31] Nies AT, Jedlitschky G, Konig J, *et al.* Expression and immunolocalization of the multidrug resistance proteins, MRP1-MRP6 (ABCC1-ABCC6), in human brain. Neuroscience 2004; 129: 349-60.
[32] Choudhuri S, Klaassen CD. Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters. Int J Toxicol 2006; 25: 231-59.
[33] Yu XQ, Xue CC, Wang G, Zhou SF. Multidrug resistance associated proteins as determining factors of pharmacokinetics and pharmacodynamics of drugs. Curr Drug Metab 2007; 8: 787-802.
[34] Ishikawa T. The ATP-dependent glutathione S-conjugate export pump. Trends Biochem Sci 1992; 17: 463-8.
[35] Yamazaki M, Suzuki H, Sugiyama Y. Recent advances in carrier-mediated hepatic uptake and biliary excretion of xenobiotics. Pharm Res 1996; 13: 497-513.
[36] Hermann DM, Bassetti CL. Implications of ATP-binding cassette transporters for brain pharmacotherapies. Trends Pharmacol Sci 2007; 28: 128-34.
[37] Vahakangas K, Myllynen P. Drug transporters in the human blood-placental barrier. Br J Pharmacol 2009; 158: 665-78.
[38] Roulet A, Puel O, Gesta S, *et al.* MDR1-deficient genotype in Collie dogs hypersensitive to the P-glycoprotein substrate ivermectin. Eur J Pharmacol 2003; 460: 85-91.
[39] Seral C, Carryn S, Tulkens PM, Van Bambeke F. Influence of P-glycoprotein and MRP efflux pump inhibitors on the intracellular activity of azithromycin and ciprofloxacin in macrophages infected by Listeria monocytogenes or *Staphylococcus aureus*. J Antimicrob Chemother 2003; 51: 1167-73.
[40] Lemaire S, Van Bambeke F, Mingeot-Leclercq MP, Tulkens PM. Modulation of the cellular accumulation and intracellular activity of daptomycin towards phagocytized *Staphylococcus aureus* by the P-glycoprotein (MDR1) efflux transporter in human THP-1 macrophages and madin-darby canine kidney cells. Antimicrob Agents Chemother 2007; 51: 2748-57.
[41] Jorajuria S, Dereuddre-Bosquet N, Becher F, *et al.* ATP binding cassette multidrug transporters limit the anti-HIV activity of zidovudine and indinavir in infected human macrophages. Antivir Ther 2004; 9: 519-28.
[42] Gillet JP, Efferth T, Remacle J. Chemotherapy-induced resistance by ATP-binding cassette transporter genes. Biochim Biophys Acta 2007; 1775: 237-62.
[43] Lage H. An overview of cancer multidrug resistance: a still unsolved problem. Cell Mol Life Sci 2008; 65: 3145-67.
[44] Reid G, Wielinga P, Zelcer N, *et al.* Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5. Mol Pharmacol 2003; 63: 1094-103.
[45] Borst P, de Wolf C, van de Wetering K. Multidrug resistance-associated proteins 3, 4, and 5. Pflugers Arch 2007; 453: 661-73.
[46] Russel FG, Koenderink JB, Masereeuw R. Multidrug resistance protein 4 (MRP4/ABCC4): a versatile efflux transporter for drugs and signalling molecules. Trends Pharmacol Sci 2008; 29: 200-7.
[47] Ernst R, Kueppers P, Stindt J, Kuchler K, Schmitt L. Multidrug efflux pumps: Substrate selection in ATP-binding cassette multidrug efflux pumps - first come, first served? FEBS J 2010; 277: 540-9.
[48] Ekins S, Ecker GF, Chiba P, Swaan PW. Future directions for drug transporter modelling. Xenobiotica 2007; 37: 1152-70.
[49] Chang C, Ekins S, Bahadduri P, Swaan PW. Pharmacophore-based discovery of ligands for drug transporters. Adv Drug Deliv Rev 2006; 58: 1431-50.
[50] Langer T, Eder M, Hoffmann RD, Chiba P, Ecker GF. Lead identification for modulators of multidrug resistance based on in silico screening with a pharmacophoric feature model. Arch Pharm (Weinheim) 2004; 337: 317-27.
[51] Martin C, Berridge G, Higgins CF, *et al.* Communication between multiple drug binding sites on P-glycoprotein. Mol Pharmacol 2000; 58: 624-32.
[52] Garrigues A, Loiseau N, Delaforge M, *et al.* Characterization of two pharmacophores on the multidrug transporter P-glycoprotein. Mol Pharmacol 2002; 62: 1288-98.
[53] Safa AR. Identification and characterization of the binding sites of P-glycoprotein for multidrug resistance-related drugs and modulators. Curr Med Chem Anticancer Agents 2004; 4: 1-17.
[54] Zelcer N, Huisman MT, Reid G, *et al.* Evidence for two interacting ligand binding sites in human multidrug resistance protein 2 (ATP binding cassette C2). J Biol Chem 2003; 278: 23538-44.
[55] Clark R, Kerr ID, Callaghan R. Multiple drug binding sites on the R482G isoform of the ABCG2 transporter. Br J Pharmacol 2006; 149: 506-15.
[56] Vandevuer S, Van Bambeke F, Tulkens PM, Prevost M. Predicting the three-dimensional structure of human P-glycoprotein in absence of ATP by computational techniques embodying crosslinking data: insight into the mechanism of ligand migration and binding sites. Proteins 2006; 63: 466-78.
[57] Michot JM, Seral C, Van Bambeke F, Mingeot-Leclercq MP, Tulkens PM. Influence of efflux transporters on the accumulation and efflux of four quinolones (ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin) in J774 macrophages. Antimicrob Agents Chemother 2005; 49: 2429-37.
[58] Marquez B, Caceres NE, Mingeot-Leclercq MP, Tulkens PM, Van Bambeke F. Identification of the efflux transporter of the fluoroquinolone antibiotic ciprofloxacin in murine macrophages: studies with ciprofloxacin-resistant cells. Antimicrob Agents Chemother 2009; 53: 2410-6.
[59] Yoshida H, Bogaki M, Nakamura S, Ubukata K, Konno M. Nucleotide sequence and characterization of the Staphylococcus aureus norA gene, which confers resistance to quinolones. J Bacteriol 1990; 172: 6942-9.
[60] Kosowska-Shick K, Credito K, Pankuch GA, *et al.* Antipneumococcal activity of DW-224a, a new quinolone, compared to those of eight other agents. Antimicrob Agents Chemother 2006; 50: 2064-71.
[61] Lismond A, Tulkens PM, Mingeot-Leclercq MP, Courvalin P, Van Bambeke F. Cooperation between prokaryotic (Lde) and eukaryotic (MRP) efflux transporters in J774 macrophages infected with Listeria monocytogenes: studies with ciprofloxacin and moxifloxacin. Antimicrob Agents Chemother 2008; 52: 3040-6.
[62] Robey RW, Honjo Y, Morisaki K, *et al.* Mutations at amino-acid 482 in the ABCG2 gene affect substrate and antagonist specificity. Br J Cancer 2003; 89: 1971-8.
[63] Gros P, Dhir R, Croop J, Talbot F. A single amino acid substitution strongly modulates the activity and substrate specificity of the mouse mdr1 and mdr3 drug efflux pumps. Proc Natl Acad Sci USA 1991; 88: 7289-93.
[64] Lown KS, Mayo RR, Leichtman AB, *et al.* Role of intestinal P-glycoprotein (mdr1) in interpatient variation in the oral bioavailability of cyclosporine. Clin Pharmacol Ther 1997; 62: 248-60.
[65] Schuetz EG, Furuya KN, Schuetz JD. Interindividual variation in expression of P-glycoprotein in normal human liver and secondary hepatic neoplasms. J Pharmacol Exp Ther 1995; 275: 1011-8.
[66] Haufroid V. Genetic polymorphisms of ATP-binding cassette transporters ABCB1 and ABCC2 and their impact on drug disposition. Curr Drug Targets 2011; 12(5): 631-46.
[67] Cusatis G, Sparreboom A. Pharmacogenomic importance of ABCG2. Pharmacogenomics 2008; 9: 1005-9.
[68] Allen JD, Brinkhuis RF, Wijnholds J, Schinkel AH. The mouse Bcrp1/Mxr/Abcp gene: amplification and overexpression in cell lines selected for resistance to topotecan, mitoxantrone, or doxorubicin. Cancer Res 1999; 59: 4237-41.
[69] Li M, Yuan H, Li N, *et al.* Identification of interspecies difference in efflux transporters of hepatocytes from dog, rat, monkey and human. Eur J Pharm Sci 2008; 35: 114-26.
[70] Zimmermann C, van de Wetering K, van de Steeg E, *et al.* Species-dependent transport and modulation properties of human and

mouse multidrug resistance protein 2 (MRP2/Mrp2, ABCC2/Abcc2). Drug Metab Dispos 2008; 36: 631-40.

[71] Ito K. ABCC2/Abcc2 transport property in different species and its modulation by heterogeneous factors. Drug Metab Pharmacokinet 2008; 23: 394-405.

[72] Syvanen S, Lindhe O, Palner M, *et al.* Species differences in blood-brain barrier transport of three positron emission tomography radioligands with emphasis on P-glycoprotein transport. Drug Metab Dispos 2009; 37: 635-43.

[73] Katoh M, Suzuyama N, Takeuchi T, *et al.* Kinetic analyses for species differences in P-glycoprotein-mediated drug transport. J Pharm Sci 2006; 95: 2673-83.

[74] Chan LM, Lowes S, Hirst BH. The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. Eur J Pharm Sci 2004; 21: 25-51.

[75] Oostendorp RL, Beijnen JH, Schellens JH. The biological and clinical role of drug transporters at the intestinal barrier. Cancer Treat Rev 2009; 35: 137-47.

[76] Mouly S, Paine MF. P-glycoprotein increases from proximal to distal regions of human small intestine. Pharm Res 2003; 20: 1595-9.

[77] Englund G, Rorsman F, Ronnblom A, *et al.* Regional levels of drug transporters along the human intestinal tract: co-expression of ABC and SLC transporters and comparison with Caco-2 cells. Eur J Pharm Sci 2006; 29: 269-77.

[78] Mottino AD, Hoffman T, Jennes L, Vore M. Expression and localization of multidrug resistant protein mrp2 in rat small intestine. J Pharmacol Exp Ther 2000; 293: 717-23.

[79] Fricker G, Drewe J, Huwyler J, Gutmann H, Beglinger C. Relevance of p-glycoprotein for the enteral absorption of cyclosporin A: *in vitro-in vivo* correlation. Br J Pharmacol 1996; 118: 1841-7.

[80] Fromm MF. Importance of P-glycoprotein for drug disposition in humans. Eur J Clin Invest 2003; 33(Suppl 2): 6-9.

[81] Jonker JW, Smit JW, Brinkhuis RF, *et al.* Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. J Natl Cancer Inst 2000; 92: 1651-6.

[82] van Herwaarden AE, Jonker JW, Wagenaar E, *et al.* The breast cancer resistance protein (Bcrp1/Abcg2) restricts exposure to the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. Cancer Res 2003; 63: 6447-52.

[83] Jonker JW, Buitelaar M, Wagenaar E, *et al.* The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. Proc Natl Acad Sci USA 2002; 99: 15649-54.

[84] Chanteux H, Van Bambeke F, Mingeot-Leclercq MP, Tulkens PM. Accumulation and oriented transport of ampicillin in Caco-2 cells from its pivaloyloxymethylester prodrug, pivampicillin. Antimicrob Agents Chemother 2005; 49: 1279-88.

[85] Ming X, Thakker DR. Role of basolateral efflux transporter MRP4 in the intestinal absorption of the antiviral drug adefovir dipivoxil. Biochem Pharmacol 2010; 79: 455-62.

[86] Hunter J, Jepson MA, Tsuruo T, Simmons NL, Hirst BH. Functional expression of P-glycoprotein in apical membranes of human intestinal Caco-2 cells. Kinetics of vinblastine secretion and interaction with modulators. J Biol Chem 1993; 268: 14991-7.

[87] Schinkel AH, Smit JJ, van Tellingen O, *et al.* Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell 1994; 77: 491-502.

[88] Sparreboom A, van Asperen J, Mayer U, *et al.* Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. Proc Natl Acad Sci USA 1997; 94: 2031-5.

[89] Kim RB, Fromm MF, Wandel C, *et al.* The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. J Clin Invest 1998; 101: 289-94.

[90] Igel S, Drescher S, Murdter T, *et al.* Increased absorption of digoxin from the human jejunum due to inhibition of intestinal transporter-mediated efflux. Clin Pharmacokinet 2007; 46: 777-85.

[91] Christians U. Transport proteins and intestinal metabolism: P-glycoprotein and cytochrome P4503A. Ther Drug Monit 2004; 26: 104-6.

[92] Wacher VJ, Salphati L, Benet LZ. Active secretion and enterocytic drug metabolism barriers to drug absorption. Adv Drug Deliv Rev 2001; 46: 89-102.

[93] Zhang Y, Benet LZ. The gut as a barrier to drug absorption: combined role of cytochrome P450 3A and P-glycoprotein. Clin Pharmacokinet 2001; 40: 159-68.

[94] Wacher VJ, Wu CY, Benet LZ. Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. Mol Carcinog 1995; 13: 129-34.

[95] van Waterschoot RA, Lagas JS, Wagenaar E, *et al.* Absence of both cytochrome P450 3A and P-glycoprotein dramatically increases docetaxel oral bioavailability and risk of intestinal toxicity. Cancer Res 2009; 69: 8996-9002.

[96] Endres CJ, Hsiao P, Chung FS, Unadkat JD. The role of transporters in drug interactions. Eur J Pharm Sci 2006; 27: 501-17.

[97] Dauchy S, Dutheil F, Weaver RJ, *et al.* ABC transporters, cytochromes P450 and their main transcription factors: expression at the human blood-brain barrier. J Neurochem 2008; 107: 1518-28.

[98] Scherrmann JM. Expression and function of multidrug resistance transporters at the blood-brain barriers. Expert Opin Drug Metab Toxicol 2005; 1: 233-46.

[99] Mayer U, Wagenaar E, Beijnen JH, *et al.* Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr 1a P-glycoprotein. Br J Pharmacol 1996; 119: 1038-44.

[100] Schinkel AH, Wagenaar E, van Deemter L, Mol CA, Borst P. Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. J Clin Invest 1995; 96: 1698-705.

[101] Schinkel AH, Wagenaar E, Mol CA, van Deemter L. P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. J Clin Invest 1996; 97: 2517-24.

[102] Kemper EM, van Zandbergen AE, Cleypool C, *et al.* Increased Penetration of Paclitaxel into the Brain by Inhibition of P-Glycoprotein. Clin Cancer Res 2003; 9: 2849-55.

[103] Schinkel AH. P-Glycoprotein, a gatekeeper in the blood-brain barrier. Adv Drug Deliv Rev 1999; 36: 179-94.

[104] van Asperen J, van Tellingen O, Schinkel AH, Beijnen JH. Comparative pharmacokinetics of vinblastine after a 96-hour continuous infusion in wild-type mice and mice lacking mdr1a P-glycoprotein. J Pharmacol Exp Ther 1999; 289: 329-33.

[105] Jonker JW, Wagenaar E, van Deemter L, *et al.* Role of blood-brain barrier P-glycoprotein in limiting brain accumulation and sedative side-effects of asimadoline, a peripherally acting analgaesic drug. Br J Pharmacol 1999; 127: 43-50.

[106] Nagengast WB, Munnink TH, Dijkers EC, *et al.* Multidrug resistance in oncology and beyond: from imaging of drug efflux pumps to cellular drug targets. Methods Mol Biol 2010; 596: 15-31.

[107] Elsinga PH, Hendrikse NH, Bart J, Vaalburg W, van Waarde A. PET Studies on P-glycoprotein function in the blood-brain barrier: how it affects uptake and binding of drugs within the CNS. Curr Pharm Des 2004; 10: 1493-503.

[108] Kawamura K, Yamasaki T, Yui J, *et al. In vivo* evaluation of P-glycoprotein and breast cancer resistance protein modulation in the brain using [(11)C]gefitinib. Nucl Med Biol 2009; 36: 239-46.

[109] Robey RW, To KK, Polgar O, *et al.* ABCG2: a perspective. Adv Drug Deliv Rev 2009; 61: 3-13.

[110] Breedveld P, Pluim D, Cipriani G, *et al.* The effect of Bcrp1 (Abcg2) on the *in vivo* pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients. Cancer Res 2005; 65: 2577-82.

[111] Lee YJ, Kusuhara H, Jonker JW, Schinkel AH, Sugiyama Y. Investigation of efflux transport of dehydroepiandrosterone sulfate and mitoxantrone at the mouse blood-brain barrier: a minor role of breast cancer resistance protein. J Pharmacol Exp Ther 2005; 312: 44-52.

[112] Enokizono J, Kusuhara H, Ose A, Schinkel AH, Sugiyama Y. Quantitative investigation of the role of breast cancer resistance protein (Bcrp/Abcg2) in limiting brain and testis penetration of xenobiotic compounds. Drug Metab Dispos 2008; 36: 995-1002.

[113] Zhao R, Raub TJ, Sawada GA, *et al.* Breast cancer resistance protein interacts with various compounds *in vitro*, but plays a

minor role in substrate efflux at the blood-brain barrier. Drug Metab Dispos 2009; 37: 1251-8.

[114] de Vries NA, Zhao J, Kroon E, *et al.* P-glycoprotein and breast cancer resistance protein: two dominant transporters working together in limiting the brain penetration of topotecan. Clin Cancer Res 2007; 13: 6440-9.

[115] Polli JW, Olson KL, Chism JP, *et al.* An unexpected synergist role of P-glycoprotein and breast cancer resistance protein on the central nervous system penetration of the tyrosine kinase inhibitor lapatinib (N-{3-chloro-4-[(3-fluorobenzyl)oxy]phenyl}-6-[5-({[2-(methylsulfonyl)ethy l]amino}methyl)-2-furyl]-4-quinazolinamine; GW572016). Drug Metab Dispos 2009; 37: 439-42.

[116] Cisternino S, Mercier C, Bourasset F, Roux F, Scherrmann JM. Expression, up-regulation, and transport activity of the multidrug-resistance protein Abcg2 at the mouse blood-brain barrier. Cancer Res 2004; 64: 3296-301.

[117] Leggas M, Adachi M, Scheffer GL, *et al.* Mrp4 confers resistance to topotecan and protects the brain from chemotherapy. Mol Cell Biol 2004; 24: 7612-21.

[118] Warren MS, Zerangue N, Woodford K, *et al.* Comparative gene expression profiles of ABC transporters in brain microvessel endothelial cells and brain in five species including human. Pharmacol Res 2009; 59: 404-13.

[119] Lankas GR, Wise LD, Cartwright ME, Pippert T, Umbenhauer DR. Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice. Reprod Toxicol 1998; 12: 457-63.

[120] Smit JW, Huisman MT, van Tellingen O, Wiltshire HR, Schinkel AH. Absence or pharmacological blocking of placental P-glycoprotein profoundly increases fetal drug exposure. J Clin Invest 1999; 104: 1441-7.

[121] Gulati A, Gerk PM. Role of placental ATP-binding cassette (ABC) transporters in antiretroviral therapy during pregnancy. J Pharm Sci 2009; 98: 2317-35.

[122] Drescher S, Glaeser H, Murdter T, *et al.* P-glycoprotein-mediated intestinal and biliary digoxin transport in humans. Clin Pharmacol Ther 2003; 73: 223-31.

[123] Klaassen CD, Aleksunes LM. Xenobiotic, Bile Acid, and Cholesterol Transporters: Function and Regulation. Pharmacol Rev 2010; 62: 1-96.

[124] Jonker JW, Stedman CA, Liddle C, Downes M. Hepatobiliary ABC transporters: physiology, regulation and implications for disease. Front Biosci 2009; 14: 4904-20.

[125] Funk C. The role of hepatic transporters in drug elimination. Expert Opin Drug Metab Toxicol 2008; 4: 363-79.

[126] Lagas JS, Vlaming ML, van Tellingen O, *et al.* Multidrug resistance protein 2 is an important determinant of paclitaxel pharmacokinetics. Clin Cancer Res 2006; 12: 6125-32.

[127] Lagas JS, Fan L, Wagenaar E, *et al.* P-glycoprotein (P-gp/Abcb1), Abcc2, and Abcc3 determine the pharmacokinetics of etoposide. Clin Cancer Res 2010; 16: 130-40.

[128] Tanaka Y, Kobayashi Y, Gabazza EC, *et al.* Increased renal expression of bilirubin glucuronide transporters in a rat model of obstructive jaundice. Am J Physiol Gastrointest Liver Physiol 2002; 282: G656-G662.

[129] van de Water FM, Masereeuw R, Russel FG. Function and regulation of multidrug resistance proteins (MRPs) in the renal elimination of organic anions. Drug Metab Rev 2005; 37: 443-71.

[130] Naud J, Michaud J, Leblond FA, *et al.* Effects of chronic renal failure on liver drug transporters. Drug Metab Dispos 2008; 36: 124-8.

[131] Sun H, Frassetto L, Benet LZ. Effects of renal failure on drug transport and metabolism. Pharmacol Ther 2006; 109: 1-11.

[132] Yu X, Zhang B, Xing C, *et al.* Different effect of cyclosporine and tacrolimus on renal expression of P-glycoprotein in human kidney transplantation. Transplant Proc 2008; 40: 3455-9.

[133] Aleksunes LM, Augustine LM, Scheffer GL, Cherrington NJ, Manautou JE. Renal xenobiotic transporters are differentially expressed in mice following cisplatin treatment. Toxicology 2008; 250: 82-8.

[134] Evans AM. Influence of dietary components on the gastrointestinal metabolism and transport of drugs. Ther Drug Monit 2000; 22: 131-6.

[135] Wagner D, Spahn-Langguth H, Hanafy A, Koggel A, Langguth P. Intestinal drug efflux: formulation and food effects. Adv Drug Deliv Rev 2001; 50(Suppl 1): S13-S31.

[136] Zhou SF. Drugs behave as substrates, inhibitors and inducers of human cytochrome P450 3A4. Curr Drug Metab 2008; 9: 310-22.

[137] Pal D, Mitra AK. MDR- and CYP3A4-mediated drug-drug interactions. J Neuroimmune Pharmacol 2006; 1: 323-39.

[138] van Heeswijk RP, Veldkamp A, Mulder JW, *et al.* Combination of protease inhibitors for the treatment of HIV-1-infected patients: a review of pharmacokinetics and clinical experience. Antivir Ther 2001; 6: 201-29.

[139] Aszalos A. Drug-drug interactions affected by the transporter protein, P-glycoprotein (ABCB1, MDR1) II. Clinical aspects. Drug Discov Today 2007; 12: 838-43.

[140] Storch CH, Theile D, Lindenmaier H, Haefeli WE, Weiss J. Comparison of the inhibitory activity of anti-HIV drugs on P-glycoprotein. Biochem Pharmacol 2007; 73: 1573-81.

[141] Nozaki Y, Kusuhara H, Kondo T, *et al.* Species difference in the inhibitory effect of nonsteroidal anti-inflammatory drugs on the uptake of methotrexate by human kidney slices. J Pharmacol Exp Ther 2007; 322: 1162-70.

[142] Lagas JS, van der Kruijssen CM, van de Wetering K, Beijnen JH, Schinkel AH. Transport of diclofenac by breast cancer resistance protein (ABCG2) and stimulation of multidrug resistance protein 2 (ABCC2)-mediated drug transport by diclofenac and benzbromarone. Drug Metab Dispos 2009; 37: 129-36.

[143] Lee CH. Reversing agents for ATP-binding cassette drug transporters. Methods Mol Biol 2010; 596: 325-40.

[144] Nobili S, Landini I, Giglioni B, Mini E. Pharmacological strategies for overcoming multidrug resistance. Curr Drug Targets 2006; 7: 861-79.

[145] Fromm MF, Kim RB, Stein CM, Wilkinson GR, Roden DM. Inhibition of P-glycoprotein-mediated drug transport: A unifying mechanism to explain the interaction between digoxin and quinidine. Circulation 1999; 99: 552-7.

[146] Westphal K, Weinbrenner A, Giessmann T, *et al.* Oral bioavailability of digoxin is enhanced by talinolol: evidence for involvement of intestinal P-glycoprotein. Clin Pharmacol Ther 2000; 68: 6-12.

[147] Rengelshausen J, Goggelmann C, Burhenne J, *et al.* Contribution of increased oral bioavailability and reduced nonglomerular renal clearance of digoxin to the digoxin-clarithromycin interaction. Br J Clin Pharmacol 2003; 56: 32-8.

[148] Verschraagen M, Koks CH, Schellens JH, Beijnen JH. P-glycoprotein system as a determinant of drug interactions: the case of digoxin-verapamil. Pharmacol Res 1999; 40: 301-6.

[149] Sadeque AJ, Wandel C, He H, Shah S, Wood AJ. Increased drug delivery to the brain by P-glycoprotein inhibition. Clin Pharmacol Ther 2000; 68: 231-7.

[150] van Asperen J, van Tellingen O, van der Valk MA, Rozenhart M, Beijnen JH. Enhanced oral absorption and decreased elimination of paclitaxel in mice cotreated with cyclosporin A. Clin Cancer Res 1998; 4: 2293-7.

[151] Meerum Terwogt JM, Malingre MM, Beijnen JH, *et al.* Coadministration of oral cyclosporin A enables oral therapy with paclitaxel. Clin Cancer Res 1999; 5: 3379-84.

[152] Malingré MM, Richel DJ, Beijnen JH, *et al.* Coadministration of cyclosporine strongly enhances the oral bioavailability of docetaxel. J Clin Oncol 2001; 19: 1160-6.

[153] Chester JD, Joel SP, Cheeseman SL, *et al.* Phase I and pharmacokinetic study of intravenous irinotecan plus oral ciclosporin in patients with fuorouracil-refractory metastatic colon cancer. J Clin Oncol 2003; 21: 1125-32.

[154] Innocenti F, Undevia SD, Ramirez J, *et al.* A phase I trial of pharmacologic modulation of irinotecan with cyclosporine and phenobarbital. Clin Pharmacol Ther 2004; 76: 490-502.

[155] Gupta A, Dai Y, Vethanayagam RR, *et al.* Cyclosporin A, tacrolimus and sirolimus are potent inhibitors of the human breast cancer resistance protein (ABCG2) and reverse resistance to mitoxantrone and topotecan. Cancer Chemother Pharmacol 2006; 58: 374-83.

[156] Qadir M, O'Loughlin KL, Fricke SM, *et al.* Cyclosporin A is a broad-spectrum multidrug resistance modulator. Clin Cancer Res 2005; 11: 2320-6.

[157] Ding R, Tayrouz Y, Riedel KD, *et al.* Substantial pharmacokinetic interaction between digoxin and ritonavir in healthy volunteers. Clin Pharmacol Ther 2004; 76: 73-84.

[158] Barau C, Blouin P, Creput C, *et al.* Effect of coadministered HIV-protease inhibitors on tacrolimus and sirolimus blood

concentrations in a kidney transplant recipient. Fundam Clin Pharmacol 2009; 23: 423-5.

[159] van Heeswijk RP, Bourbeau M, Campbell P, *et al.* Time-dependent interaction between lopinavir/ritonavir and fexofenadine. J Clin Pharmacol 2006; 46: 758-67.

[160] Mukwaya G, MacGregor T, Hoelscher D, *et al.* Interaction of ritonavir-boosted tipranavir with loperamide does not result in loperamide-associated neurologic side effects in healthy volunteers. Antimicrob Agents Chemother 2005; 49: 4903-10.

[161] Vourvahis M, Kashuba AD. Mechanisms of pharmacokinetic and pharmacodynamic drug interactions associated with ritonavir-enhanced tipranavir. Pharmacotherapy 2007; 27: 888-909.

[162] Gupta A, Zhang Y, Unadkat JD, Mao Q. HIV protease inhibitors are inhibitors but not substrates of the human breast cancer resistance protein (BCRP/ABCG2). J Pharmacol Exp Ther 2004; 310: 334-41.

[163] Weiss J, Rose J, Storch CH, *et al.* Modulation of human BCRP (ABCG2) activity by anti-HIV drugs. J Antimicrob Chemother 2007; 59: 238-45.

[164] Gupta A, Unadkat JD, Mao Q. Interactions of azole antifungal agents with the human breast cancer resistance protein (BCRP). J Pharm Sci 2007; 96: 3226-35.

[165] Breedveld P, Zelcer N, Pluim D, *et al.* Mechanism of the pharmacokinetic interaction between methotrexate and benzimidazoles: potential role for breast cancer resistance protein in clinical drug-drug interactions. Cancer Res 2004; 64: 5804-11.

[166] Tracy TS, Krohn K, Jones DR, *et al.* The effects of a salicylate, ibuprofen, and naproxen on the disposition of methotrexate in patients with rheumatoid arthritis. Eur J Clin Pharmacol 1992; 42: 121-5.

[167] El Sheikh AA, van den Heuvel JJ, Koenderink JB, Russel FG. Interaction of nonsteroidal anti-inflammatory drugs with multidrug resistance protein (MRP) 2/ABCC2- and MRP4/ABCC4-mediated methotrexate transport. J Pharmacol Exp Ther 2007; 320: 229-35.

[168] Vautier S, Milane A, Fernandez C, *et al.* Interactions between antiparkinsonian drugs and ABCB1/P-glycoprotein at the blood-brain barrier in a rat brain endothelial cell model. Neurosci Lett 2008; 442: 19-23.

[169] Kakumoto M, Takara K, Sakaeda T, *et al.* MDR1-mediated interaction of digoxin with antiarrhythmic or antianginal drugs. Biol Pharm Bull 2002; 25: 1604-7.

[170] Terashi K, Oka M, Soda H, *et al.* Interactions of ofloxacin and erythromycin with the multidrug resistance protein (MRP) in MRP-overexpressing human leukemia cells. Antimicrob Agents Chemother 2000; 44: 1697-700.

[171] Hemauer SJ, Patrikeeva SL, Nanovskaya TN, Hankins GD, Ahmed MS. Opiates inhibit paclitaxel uptake by P-glycoprotein in preparations of human placental inside-out vesicles. Biochem Pharmacol 2009; 78: 1272-8.

[172] Klaassen CD, Slitt AL. Regulation of hepatic transporters by xenobiotic receptors. Curr Drug Metab 2005; 6: 309-28.

[173] Urquhart BL, Tirona RG, Kim RB. Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs. J Clin Pharmacol 2007; 47: 566-78.

[174] Kast HR, Goodwin B, Tarr PT, *et al.* Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. J Biol Chem 2002; 277: 2908-15.

[175] Teng S, Jekerle V, Piquette-Miller M. Induction of ABCC3 (MRP3) by pregnane X receptor activators. Drug Metab Dispos 2003; 31: 1296-9.

[176] Assem M, Schuetz EG, Leggas M, *et al.* Interactions between hepatic Mrp4 and Sult2a as revealed by the constitutive androstane receptor and Mrp4 knockout mice. J Biol Chem 2004; 279: 22250-7.

[177] Szatmari I, Vamosi G, Brazda P, *et al.* Peroxisome proliferator-activated receptor gamma-regulated ABCG2 expression confers cytoprotection to human dendritic cells. J Biol Chem 2006; 281: 23812-23.

[178] Harmsen S, Meijerman I, Beijnen JH, Schellens JH. The role of nuclear receptors in pharmacokinetic drug-drug interactions in oncology. Cancer Treat Rev 2007; 33: 369-80.

[179] Cheng X, Maher J, Dieter MZ, Klaassen CD. Regulation of mouse organic anion-transporting polypeptides (Oatps) in liver by prototypical microsomal enzyme inducers that activate distinct transcription factor pathways. Drug Metab Dispos 2005; 33: 1276-82.

[180] Timsit YE, Negishi M. CAR and PXR: the xenobiotic-sensing receptors. Steroids 2007; 72: 231-46.

[181] Schuetz EG, Beck WT, Schuetz JD. Modulators and substrates of P-glycoprotein and cytochrome P4503A coordinately up-regulate these proteins in human colon carcinoma cells. Mol Pharmacol 1996; 49: 311-8.

[182] Rao VK, Wangsa D, Robey RW, *et al.* Characterization of ABCG2 gene amplification manifesting as extrachromosomal DNA in mitoxantrone-selected SF295 human glioblastoma cells. Cancer Genet Cytogenet 2005; 160: 126-33.

[183] Fromm MF, Kauffmann HM, Fritz P, *et al.* The effect of rifampin treatment on intestinal expression of human MRP transporters. Am J Pathol 2000; 157: 1575-80.

[184] Greiner B, Eichelbaum M, Fritz P, *et al.* The role of intestinal P-glycoprotein in the interaction of digoxin and rifampin. J Clin Invest 1999; 104: 147-53.

[185] Geick A, Eichelbaum M, Burk O. Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. J Biol Chem 2001; 276: 14581-7.

[186] Westphal K, Weinbrenner A, Zschiesche M, *et al.* Induction of P-glycoprotein by rifampin increases intestinal secretion of talinolol in human beings: a new type of drug/drug interaction. Clin Pharmacol Ther 2000; 68: 345-55.

[187] Hamman MA, Bruce MA, Haehner-Daniels BD, Hall SD. The effect of rifampin administration on the disposition of fexofenadine. Clin Pharmacol Ther 2001; 69: 114-21.

[188] Hebert MF, Roberts JP, Prueksaritanont T, Benet LZ. Bioavailability of cyclosporine with concomitant rifampin administration is markedly less than predicted by hepatic enzyme induction. Clin Pharmacol Ther 1992; 52: 453-7.

[189] Bauer B, Yang X, Hartz AM, *et al. In vivo* activation of human pregnane X receptor tightens the blood-brain barrier to methadone through P-glycoprotein up-regulation. Mol Pharmacol 2006; 70: 1212-9.

[190] Huang L, Wring SA, Woolley JL, *et al.* Induction of P-glycoprotein and cytochrome P450 3A by HIV protease inhibitors. Drug Metab Dispos 2001; 29: 754-60.

[191] Perloff MD, von Moltke LL, Marchand JE, Greenblatt DJ. Ritonavir induces P-glycoprotein expression, multidrug resistance-associated protein (MRP1) expression, and drug transporter-mediated activity in a human intestinal cell line. J Pharm Sci 2001; 90: 1829-37.

[192] Perloff MD, von Moltke LL, Greenblatt DJ. Ritonavir and dexamethasone induce expression of CYP3A and P-glycoprotein in rats. Xenobiotica 2004; 34: 133-50.

[193] Perloff ES, Duan SX, Skolnik PR, Greenblatt DJ, von Moltke LL. Atazanavir: effects on P-glycoprotein transport and CYP3A metabolism *in vitro*. Drug Metab Dispos 2005; 33: 764-70.

[194] Zastre JA, Chan GN, Ronaldson PT, *et al.* Up-regulation of P-glycoprotein by HIV protease inhibitors in a human brain microvessel endothelial cell line. J Neurosci Res 2009; 87: 1023-36.

[195] Chandler B, Almond L, Ford J, *et al.* The effects of protease inhibitors and nonnucleoside reverse transcriptase inhibitors on p-glycoprotein expression in peripheral blood mononuclear cells *in vitro*. J Acquir Immune Defic Syndr 2003; 33: 551-6.

[196] Dussault I, Lin M, Hollister K, *et al.* Peptide mimetic HIV protease inhibitors are ligands for the orphan receptor SXR. J Biol Chem 2001; 276: 33309-12.

[197] Ford J, Meaden ER, Hoggard PG, *et al.* Effect of protease inhibitor-containing regimens on lymphocyte multidrug resistance transporter expression. J Antimicrob Chemother 2003; 52: 354-8.

[198] Weiss J, Weis N, Ketabi-Kiyanvash N, Storch CH, Haefeli WE. Comparison of the induction of P-glycoprotein activity by nucleotide, nucleoside, and non-nucleoside reverse transcriptase inhibitors. Eur J Pharmacol 2008; 579: 104-9.

[199] Stormer E, von Moltke LL, Perloff MD, Greenblatt DJ. Differential modulation of P-glycoprotein expression and activity by non-nucleoside HIV-1 reverse transcriptase inhibitors in cell culture. Pharm Res 2002; 19: 1038-45.

[200] Hassan HE, Myers AL, Lee IJ, Coop A, Eddington ND. Oxycodone induces overexpression of P-glycoprotein (ABCB1) and affects paclitaxel's tissue distribution in Sprague Dawley rats. J Pharm Sci 2007; 96: 2494-506.

[201] Gradilone A, Pulcinelli FM, Lotti LV, *et al*. Celecoxib upregulates multidrug resistance proteins in colon cancer: lack of synergy with standard chemotherapy. Curr Cancer Drug Targets 2008; 8: 414-20.
[202] Xu Y, Kolesar JM, Schaaf LJ, *et al.* Phase I and pharmacokinetic study of mitomycin C and celecoxib as potential modulators of tumor resistance to irinotecan in patients with solid malignancies. Cancer Chemother Pharmacol 2009; 63: 1073-82.
[203] Giessmann T, May K, Modess C, *et al.* Carbamazepine regulates intestinal P-glycoprotein and multidrug resistance protein MRP2 and influences disposition of talinolol in humans. Clin Pharmacol Ther 2004; 76: 192-200.
[204] Yang HW, Liu HY, Liu X, *et al.* Increased P-glycoprotein function and level after long-term exposure of four antiepileptic drugs to rat brain microvascular endothelial cells *in vitro*. Neurosci Lett 2008; 434: 299-303.
[205] Lombardo L, Pellitteri R, Balazy M, Cardile V. Induction of nuclear receptors and drug resistance in the brain microvascular endothelial cells treated with antiepileptic drugs. Curr Neurovasc Res 2008; 5: 82-92.
[206] Wen T, Liu YC, Yang HW, *et al.* Effect of 21-day exposure of phenobarbital, carbamazepine and phenytoin on P-glycoprotein expression and activity in the rat brain. J Neurol Sci 2008; 270: 99-106.
[207] Michot JM, Heremans MF, Caceres NE, *et al.* Cellular accumulation and activity of quinolones in ciprofloxacin-resistant J774 macrophages. Antimicrob Agents Chemother 2006; 50: 1689-95.
[208] Hatse S, De Clercq E, Balzarini J. Enhanced 9-(2-phosphonylmethoxyethyl)adenine secretion by a specific, indomethacin-sensitive efflux pump in a mutant 9-(2-phosphonylmethoxyethyl) adenine-resistant human erythroleukemia K562 cell line. Mol Pharmacol 1998; 54: 907-17.
[209] Gottesman MM, Cardarelli C, Goldenberg S, Licht T, Pastan I. Selection and maintenance of multidrug-resistant cells. Methods Enzymol 1998; 292: 248-58.
[210] Tada Y, Wada M, Migita T, *et al.* Increased expression of multidrug resistance-associated proteins in bladder cancer during clinical course and drug resistance to doxorubicin. Int J Cancer 2002; 98: 630-5.
[211] Ross DD, Nakanishi T. Impact of breast cancer resistance protein on cancer treatment outcomes. Methods Mol Biol 2010; 596: 251-90.
[212] van den Heuvel-Eibrink MM, Sonneveld P, Pieters R. The prognostic significance of membrane transport-associated multidrug resistance (MDR) proteins in leukemia. Int J Clin Pharmacol Ther 2000; 38: 94-110.
[213] Lazarowski A, Czornyj L, Lubienieki F, *et al.* ABC transporters during epilepsy and mechanisms underlying multidrug resistance in refractory epilepsy. Epilepsia 2007; 48(Suppl 5): 140-9.
[214] Loscher W, Potschka H. Drug resistance in brain diseases and the role of drug efflux transporters. Nat Rev Neurosci 2005; 6: 591-602.
[215] Baumert C, Hilgeroth A. Recent advances in the development of P-gp inhibitors. Anticancer Agents Med Chem 2009; 9: 415-36.
[216] Shukla S, Ohnuma S, Ambudkar SV. Improving cancer chemotherapy with modulators of ABC drug transporters. Curr Drug Targets 2011; 12(5): 621-30.
[217] Zhang L, Zhang YD, Strong JM, Reynolds KS, Huang SM. A regulatory viewpoint on transporter-based drug interactions. Xenobiotica 2008; 38: 709-24.
[218] Zhang L, Zhang YD, Zhao P, Huang SM. Predicting drug-drug interactions: an FDA perspective. AAPS J 2009; 11: 300-6.
[219] US Food and Drug Administration Guidance for Industry: Drug Interaction Studies — Study Design, Data Analysis, and Implications for Dosing and Labeling. Available at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072101.pdf [accessed 8-2-2010].
[220] Cordon-Cardo C, O'Brien JP, Boccia J, *et al.* Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. J Histochem Cytochem 1990; 38: 1277-87.
[221] Roelofsen H, Vos TA, Schippers IJ, *et al.* Increased levels of the multidrug resistance protein in lateral membranes of proliferating hepatocyte-derived cells. Gastroenterology 1997; 112: 511-21.
[222] St Pierre MV, Serrano MA, Macias RI, *et al.* Expression of members of the multidrug resistance protein family in human term placenta. Am J Physiol Regul Integr Comp Physiol 2000; 279: R1495-R1503.
[223] Flens MJ, Zaman GJ, van der Valk P, *et al.* Tissue distribution of the multidrug resistance protein. Am J Pathol 1996; 148: 1237-47.
[224] Schaub TP, Kartenbeck J, Konig J, *et al.* Expression of the MRP2 gene-encoded conjugate export pump in human kidney proximal tubules and in renal cell carcinoma. J Am Soc Nephrol 1999; 10: 1159-69.
[225] Kartenbeck J, Leuschner U, Mayer R, Keppler D. Absence of the canalicular isoform of the MRP gene-encoded conjugate export pump from the hepatocytes in Dubin-Johnson syndrome. Hepatology 1996; 23: 1061-6.
[226] Scheffer GL, Kool M, de Haas M, *et al.* Tissue distribution and induction of human multidrug resistant protein 3. Lab Invest 2002; 82: 193-201.
[227] Belinsky MG, Bain LJ, Balsara BB, Testa JR, Kruh GD. Characterization of MOAT-C and MOAT-D, new members of the MRP/cMOAT subfamily of transporter proteins. J Natl Cancer Inst 1998; 90: 1735-41.
[228] Lee K, Klein-Szanto AJ, Kruh GD. Analysis of the MRP4 drug resistance profile in transfected NIH3T3 cells. J Natl Cancer Inst 2000; 92: 1934-40.
[229] van Aubel RA, Smeets PH, Peters JG, Bindels RJ, Russel FG. The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. J Am Soc Nephrol 2002; 13: 595-603.
[230] Rius M, Nies AT, Hummel-Eisenbeiss J, Jedlitschky G, Keppler D. Cotransport of reduced glutathione with bile salts by MRP4 (ABCC4) localized to the basolateral hepatocyte membrane. Hepatology 2003; 38: 374-84.
[231] Konig J, Hartel M, Nies AT, *et al.* Expression and localization of human multidrug resistance protein (ABCC) family members in pancreatic carcinoma. Int J Cancer 2005; 115: 359-67.
[232] Lee K, Belinsky MG, Bell DW, Testa JR, Kruh GD. Isolation of MOAT-B, a widely expressed multidrug resistance-associated protein/canalicular multispecific organic anion transporter-related transporter. Cancer Res 1998; 58: 2741-7.
[233] Scheffer GL, Hu X, Pijnenborg AC, *et al.* MRP6 (ABCC6) detection in normal human tissues and tumors. Lab Invest 2002; 82: 515-8.
[234] Beck K, Hayashi K, Dang K, Hayashi M, Boyd CD. Analysis of ABCC6 (MRP6) in normal human tissues. Histochem Cell Biol 2005; 123: 517-28.
[235] Hopper E, Belinsky MG, Zeng H, *et al.* Analysis of the structure and expression pattern of MRP7 (ABCC10), a new member of the MRP subfamily. Cancer Lett 2001; 162: 181-91.
[236] Takayanagi S, Kataoka T, Ohara O, *et al.* Human ATP-binding cassette transporter ABCC10: expression profile and p53-dependent upregulation. J Exp Ther Oncol 2004; 4: 239-46.
[237] Maliepaard M, Scheffer GL, Faneyte IF, *et al.* Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. Cancer Res 2001; 61: 3458-64.
[238] Scheffer GL, Pijnenborg AC, Smit EF, *et al.* Multidrug resistance related molecules in human and murine lung. J Clin Pathol 2002; 55: 332-9.
[239] Zimmermann C, Hruz P, Gutmann H, *et al.* Decreased expression of breast cancer resistance protein in the duodenum in patients with obstructive cholestasis. Digestion 2006; 74: 101-8.
[240] Doyle LA, Ross DD. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene 2003; 22: 7340-58.
[241] Pavek P, Merino G, Wagenaar E, *et al.* Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine. J Pharmacol Exp Ther 2005; 312: 144-52.
[242] Collett A, Higgs NB, Sims E, Rowland M, Warhurst G. Modulation of the permeability of H2 receptor antagonists cimetidine and ranitidine by P-glycoprotein in rat intestine and the human colonic cell line Caco-2. J Pharmacol Exp Ther 1999; 288: 171-8.
[243] Dahan A, Sabit H, Amidon GL. The H2 receptor antagonist nizatidine is a P-glycoprotein substrate: characterization of its intestinal epithelial cell efflux transport. AAPS J 2009; 11: 205-13.

[244] Spahn-Langguth H, Baktir G, Radschuweit A, *et al.* P-glycoprotein transporters and the gastrointestinal tract: evaluation of the potential *in vivo* relevance of *in vitro* data employing talinolol as model compound. Int J Clin Pharmacol Ther 1998; 36: 16-24.

[245] Zhou L, Schmidt K, Nelson FR, *et al.* The effect of breast cancer resistance protein and P-glycoprotein on the brain penetration of flavopiridol, imatinib mesylate (Gleevec), prazosin, and 2-methoxy-3-(4-(2-(5-methyl-2-phenyloxazol-4-yl)ethoxy)phenyl) propanoic acid (PF-407288) in mice. Drug Metab Dispos 2009; 37: 946-55.

[246] Karlsson J, Kuo SM, Ziemniak J, Artursson P. Transport of celiprolol across human intestinal epithelial (Caco-2) cells: mediation of secretion by multiple transporters including P-glycoprotein. Br J Pharmacol 1993; 110: 1009-16.

[247] Shukla S, Robey RW, Bates SE, Ambudkar SV. The calcium channel blockers, 1,4-dihydropyridines, are substrates of the multidrug resistance-linked ABC drug transporter, ABCG2. Biochemistry 2006; 45: 8940-51.

[248] Takara K, Sakaeda T, Tanigawara Y, *et al.* Effects of 12 Ca2+ antagonists on multidrug resistance, MDR1-mediated transport and MDR1 mRNA expression. Eur J Pharm Sci 2002; 16: 159-65.

[249] Dorababu M, Nishimura A, Prabha T, *et al.* Effect of cyclosporine on drug transport and pharmacokinetics of nifedipine. Biomed Pharmacother 2009; 63: 697-702.

[250] Saeki T, Ueda K, Tanigawara Y, Hori R, Komano T. P-glycoprotein-mediated transcellular transport of MDR-reversing agents. FEBS Lett 1993; 324: 99-102.

[251] Yusa K, Tsuruo T. Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. Cancer Res 1989; 49: 5002-6.

[252] Soldner A, Benet LZ, Mutschler E, Christians U. Active transport of the angiotensin-II antagonist losartan and its main metabolite EXP 3174 across MDCK-MDR1 and caco-2 cell monolayers. Br J Pharmacol 2000; 129: 1235-43.

[253] Oswald S, Haenisch S, Fricke C, *et al.* Intestinal expression of P-glycoprotein (ABCB1), multidrug resistance associated protein 2 (ABCC2), and uridine diphosphate-glucuronosyltransferase 1A1 predicts the disposition and modulates the effects of the cholesterol absorption inhibitor ezetimibe in humans. Clin Pharmacol Ther 2006; 79: 206-17.

[254] Hochman JH, Pudvah N, Qiu J, *et al.* Interactions of human P-glycoprotein with simvastatin, simvastatin acid, and atorvastatin. Pharm Res 2004; 21: 1686-91.

[255] Wang E, Casciano CN, Clement RP, Johnson WW. HMG-CoA reductase inhibitors (statins) characterized as direct inhibitors of P-glycoprotein. Pharm Res 2001; 18: 800-6.

[256] Bogman K, Peyer AK, Torok M, Kusters E, Drewe J. HMG-CoA reductase inhibitors and P-glycoprotein modulation. Br J Pharmacol 2001; 132: 1183-92.

[257] Hirano M, Maeda K, Matsushima S, *et al.* Involvement of BCRP (ABCG2) in the biliary excretion of pitavastatin. Mol Pharmacol 2005; 68: 800-7.

[258] Uchida Y, Kamiie J, Ohtsuki S, Terasaki T. Multichannel liquid chromatography-tandem mass spectrometry cocktail method for comprehensive substrate characterization of multidrug resistance-associated protein 4 transporter. Pharm Res 2007; 24: 2281-96.

[259] Yamazaki M, Akiyama S, Ni'inuma K, Nishigaki R, Sugiyama Y. Biliary excretion of pravastatin in rats: contribution of the excretion pathway mediated by canalicular multispecific organic anion transporter. Drug Metab Dispos 1997; 25: 1123-9.

[260] Huang L, Wang Y, Grimm S. ATP-dependent transport of rosuvastatin in membrane vesicles expressing breast cancer resistance protein. Drug Metab Dispos 2006; 34: 738-42.

[261] Kitamura S, Maeda K, Wang Y, Sugiyama Y. Involvement of multiple transporters in the hepatobiliary transport of rosuvastatin. Drug Metab Dispos 2008; 36: 2014-23.

[262] Kavallaris M, Madafiglio J, Norris MD, Haber M. Resistance to tetracycline, a hydrophilic antibiotic, is mediated by P-glycoprotein in human multidrug-resistant cells. Biochem Biophys Res Commun 1993; 190: 79-85.

[263] Ci L, Kusuhara H, Adachi M, *et al.* Involvement of MRP4 (ABCC4) in the luminal efflux of ceftizoxime and cefazolin in the kidney. Mol Pharmacol 2007; 71: 1591-7.

[264] Kato Y, Takahara S, Kato S, *et al.* Involvement of multidrug resistance-associated protein 2 (Abcc2) in molecular weight-dependent biliary excretion of beta-lactam antibiotics. Drug Metab Dispos 2008; 36: 1088-96.

[265] Susanto M, Benet LZ. Can the enhanced renal clearance of antibiotics in cystic fibrosis patients be explained by P-glycoprotein transport? Pharm Res 2002; 19: 457-62.

[266] Sugie M, Asakura E, Zhao YL, *et al.* Possible involvement of the drug transporters P glycoprotein and multidrug resistance-associated protein Mrp2 in disposition of azithromycin. Antimicrob Agents Chemother 2004; 48: 809-14.

[267] Takano M, Hasegawa R, Fukuda T, *et al.* Interaction with P-glycoprotein and transport of erythromycin, midazolam and ketoconazole in Caco-2 cells. Eur J Pharmacol 1998; 358: 289-94.

[268] Terashi K, Oka M, Soda H, *et al.* Interactions of ofloxacin and erythromycin with the multidrug resistance protein (MRP) in MRP-overexpressing human leukemia cells. Antimicrob Agents Chemother 2000; 44: 1697-700.

[269] Yamaguchi S, Zhao YL, Nadai M, *et al.* Involvement of the drug transporters P-glycoprotein and multidrug resistance-associated protein Mrp2 in telithromycin transport. Antimicrob Agents Chemother 2006; 50: 80-7.

[270] Ito T, Yano I, Tanaka K, Inui KI. Transport of quinolone antibacterial drugs by human P-glycoprotein expressed in a kidney epithelial cell line, LLC-PK1. J Pharmacol Exp Ther 1997; 282: 955-60.

[271] Cormet-Boyaka E, Huneau JF, Mordrelle A, *et al.* Secretion of sparfloxacin from the human intestinal Caco-2 cell line is altered by P-glycoprotein inhibitors. Antimicrob Agents Chemother 1998; 42: 2607-11.

[272] Tamai I, Yamashita J, Kido Y, *et al.* Limited distribution of new quinolone antibacterial agents into brain caused by multiple efflux transporters at the blood-brain barrier. J Pharmacol Exp Ther 2000; 295: 146-52.

[273] Naruhashi K, Tamai I, Inoue N, *et al.* Active intestinal secretion of new quinolone antimicrobials and the partial contribution of P-glycoprotein. J Pharm Pharmacol 2001; 53: 699-709.

[274] Naruhashi K, Tamai I, Inoue N, *et al.* Involvement of Multidrug Resistance-Associated Protein 2 in Intestinal Secretion of Grepafloxacin in Rats. Antimicrob Agents Chemother 2002; 46: 344-9.

[275] Ando T, Kusuhara H, Merino G, *et al.* Involvement of breast cancer resistance protein (ABCG2) in the biliary excretion mechanism of fluoroquinolones. Drug Metab Dispos 2007; 35: 1873-9.

[276] Brillault J, De Castro WV, Harnois T, *et al.* P-glycoprotein-mediated transport of moxifloxacin in a Calu-3 lung epithelial cell model. Antimicrob Agents Chemother 2009; 53: 1457-62.

[277] Merino G, Jonker JW, Wagenaar E, van Herwaarden AE, Schinkel AH. The breast cancer resistance protein (BCRP/ABCG2) affects pharmacokinetics, hepatobiliary excretion, and milk secretion of the antibiotic nitrofurantoin. Mol Pharmacol 2005; 67: 1758-64.

[278] Miyama T, Takanaga H, Matsuo H, *et al.* P-glycoprotein-mediated transport of itraconazole across the blood-brain barrier. Antimicrob Agents Chemother 1998; 42: 1738-44.

[279] Schuetz EG, Schinkel AH, Relling MV, Schuetz JD. P-glycoprotein: a major determinant of rifampicin-inducible expression of cytochrome P4503A in mice and humans. Proc Natl Acad Sci USA 1996; 93: 4001-5.

[280] Dallas S, Schlichter L, Bendayan R. Multidrug resistance protein (MRP) 4- and MRP 5-mediated efflux of 9-(2-phosphonyl-methoxyethyl)adenine by microglia. J Pharmacol Exp Ther 2004; 309: 1221-9.

[281] Hopper-Borge E, Xu X, Shen T, *et al.* Human multidrug resistance protein 7 (ABCC10) is a resistance factor for nucleoside analogues and epothilone B. Cancer Res 2009; 69: 178-84.

[282] Schuetz JD, Connelly MC, Sun D, *et al.* MRP4: A previously unidentified factor in resistance to nucleoside-based antiviral drugs. Nat Med 1999; 5: 1048-51.

[283] Takenaka K, Morgan JA, Scheffer GL, *et al.* Substrate overlap between Mrp4 and Abcg2/Bcrp affects purine analogue drug cytotoxicity and tissue distribution. Cancer Res 2007; 67: 6965-72.

[284] Adachi M, Sampath J, Lan LB, *et al.* Expression of MRP4 confers resistance to ganciclovir and compromises bystander cell killing. J Biol Chem 2002; 277: 38998-9004.

[285] Wang X, Furukawa T, Nitanda T, *et al.* Breast cancer resistance protein (BCRP/ABCG2) induces cellular resistance to HIV-1

nucleoside reverse transcriptase inhibitors. Mol Pharmacol 2003; 63: 65-72.

[286] Huisman MT, Smit JW, Crommentuyn KM, *et al.* Multidrug resistance protein 2 (MRP2) transports HIV protease inhibitors, and transport can be enhanced by other drugs. AIDS 2002; 16: 2295-301.

[287] Lee CG, Gottesman MM, Cardarelli CO, *et al.* HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter. Biochemistry 1998; 37: 3594-601.

[288] Jones K, Bray PG, Khoo SH, *et al.* P-Glycoprotein and transporter MRP1 reduce HIV protease inhibitor uptake in CD4 cells: potential for accelerated viral drug resistance? AIDS 2001; 15: 1353-8.

[289] Agarwal S, Pal D, Mitra AK. Both P-gp and MRP2 mediate transport of Lopinavir, a protease inhibitor. Int J Pharm 2007; 339: 139-47.

[290] Williams GC, Liu A, Knipp G, Sinko PJ. Direct evidence that saquinavir is transported by multidrug resistance-associated protein (MRP1) and canalicular multispecific organic anion transporter (MRP2). Antimicrob Agents Chemother 2002; 46: 3456-62.

[291] de Wolf C, Jansen R, Yamaguchi H, *et al.* Contribution of the drug transporter ABCG2 (breast cancer resistance protein) to resistance against anticancer nucleosides. Mol Cancer Ther 2008; 7: 3092-102.

[292] Norris MD, De Graaf D, Haber M, *et al.* Involvement of MDR1 P-glycoprotein in multifactorial resistance to methotrexate. Int J Cancer 1996; 65: 613-9.

[293] Chen ZS, Lee K, Walther S, *et al.* Analysis of methotrexate and folate transport by multidrug resistance protein 4 (ABCC4): MRP4 is a component of the methotrexate efflux system. Cancer Res 2002; 62: 3144-50.

[294] Zeng H, Chen ZS, Belinsky MG, Rea PA, Kruh GD. Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport. Cancer Res 2001; 61: 7225-32.

[295] Zeng H, Bain LJ, Belinsky MG, Kruh GD. Expression of multidrug resistance protein-3 (multispecific organic anion transporter-D) in human embryonic kidney 293 cells confers resistance to anticancer agents. Cancer Res 1999; 59: 5964-7.

[296] Wielinga P, Hooijberg JH, Gunnarsdottir S, *et al.* The human multidrug resistance protein MRP5 transports folates and can mediate cellular resistance against antifolates. Cancer Res 2005; 65: 4425-30.

[297] Volk EL, Farley KM, Wu Y, *et al.* Overexpression of wild-type breast cancer resistance protein mediates methotrexate resistance. Cancer Res 2002; 62: 5035-40.

[298] Hooijberg JH, Broxterman HJ, Kool M, *et al.* Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. Cancer Res 1999; 59: 2532-5.

[299] Chen ZS, Robey RW, Belinsky MG, *et al.* Transport of methotrexate, methotrexate polyglutamates, and 17beta-estradiol 17-(beta-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport. Cancer Res 2003; 63: 4048-54.

[300] Huisman MT, Chhatta AA, van Tellingen O, Beijnen JH, Schinkel AH. MRP2 (ABCC2) transports taxanes and confers paclitaxel resistance and both processes are stimulated by probenecid. Int J Cancer 2005; 116: 824-9.

[301] Hopper-Borge E, Chen ZS, Shchaveleva I, Belinsky MG, Kruh GD. Analysis of the drug resistance profile of multidrug resistance protein 7 (ABCC10): resistance to docetaxel. Cancer Res 2004; 64: 4927-30.

[302] Shirakawa K, Takara K, Tanigawara Y, *et al.* Interaction of docetaxel ("Taxotere") with human P-glycoprotein. Jpn J Cancer Res 1999; 90: 1380-6.

[303] Zaman GJ, Flens MJ, van Leusden MR, *et al.* The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. Proc Natl Acad Sci USA 1994; 91: 8822-6.

[304] Breuninger LM, Paul S, Gaughan K, *et al.* Expression of multidrug resistance-associated protein in NIH/3T3 cells confers multidrug resistance associated with increased drug efflux and altered intracellular drug distribution. Cancer Res 1995; 55: 5342-7.

[305] Bhalla K, Huang Y, Tang C, *et al.* Characterization of a human myeloid leukemia cell line highly resistant to taxol. Leukemia 1994; 8: 465-75.

[306] Kool M, van der Linden M, de Haas M, *et al.* MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci USA 1999; 96: 6914-9.

[307] Cui Y, Konig J, Buchholz JK, *et al.* Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol Pharmacol 1999; 55: 929-37.

[308] Belinsky MG, Chen ZS, Shchaveleva I, Zeng H, Kruh GD. Characterization of the drug resistance and transport properties of multidrug resistance protein 6 (MRP6, ABCC6). Cancer Res 2002; 62: 6172-7.

[309] Guo A, Marinaro W, Hu P, Sinko PJ. Delineating the contribution of secretory transporters in the efflux of etoposide using Madin-Darby canine kidney (MDCK) cells overexpressing P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP1), and canalicular multispecific organic anion transporter (cMOAT). Drug Metab Dispos 2002; 30: 457-63.

[310] Zelcer N, Saeki T, Reid G, Beijnen JH, Borst P. Characterization of drug transport by the human multidrug resistance protein 3 (ABCC3). J Biol Chem 2001; 276: 46400-7.

[311] Horio M, Gottesman MM, Pastan I. ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. Proc Natl Acad Sci USA 1988; 85: 3580-4.

[312] Evers R, Kool M, van Deemter L, *et al.* Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. J Clin Invest 1998; 101: 1310-9.

[313] Evers R, de Haas M, Sparidans R, *et al.* Vinblastine and sulfinpyrazone export by the multidrug resistance protein MRP2 is associated with glutathione export. Br J Cancer 2000; 83: 375-83.

[314] Horio M, Chin KV, Currier SJ, *et al.* Transepithelial transport of drugs by the multidrug transporter in cultured Madin-Darby canine kidney cell epithelia. J Biol Chem 1989; 264: 14880-4.

[315] Loe DW, Deeley RG, Cole SP. Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione. Cancer Res 1998; 58: 5130-6.

[316] Doyle LA, Yang W, Abruzzo LV, *et al.* A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci USA 1998; 95: 15665-70.

[317] Cole SP, Deeley RG. Transport of glutathione and glutathione conjugates by MRP1. Trends Pharmacol Sci 2006; 27: 438-46.

[318] Bellamy WT, Dalton WS, Kailey JM, *et al.* Verapamil reversal of doxorubicin resistance in multidrug-resistant human myeloma cells and association with drug accumulation and DNA damage. Cancer Res 1988; 48: 6365-70.

[319] Morrow CS, Peklak-Scott C, Bishwokarma B, *et al.* Multidrug resistance protein 1 (MRP1, ABCC1) mediates resistance to mitoxantrone via glutathione-dependent drug efflux. Mol Pharmacol 2006; 69: 1499-505.

[320] Consoli U, Van NT, Neamati N, *et al.* Cellular pharmacology of mitoxantrone in p-glycoprotein-positive and -negative human myeloid leukemic cell lines. Leukemia 1997; 11: 2066-74.

[321] Luo FR, Paranjpe PV, Guo A, Rubin E, Sinko P. Intestinal transport of irinotecan in Caco-2 cells and MDCK II cells overexpressing efflux transporters Pgp, cMOAT, and MRP1. Drug Metab Dispos 2002; 30: 763-70.

[322] Chen ZS, Furukawa T, Sumizawa T, *et al.* ATP-Dependent efflux of CPT-11 and SN-38 by the multidrug resistance protein (MRP) and its inhibition by PAK-104P. Mol Pharmacol 1999; 55: 921-8.

[323] Schellens JH, Maliepaard M, Scheper RJ, *et al.* Transport of topoisomerase I inhibitors by the breast cancer resistance protein. Potential clinical implications. Ann N Y Acad Sci 2000; 922: 188-94.

[324] Tian Q, Zhang J, Tan TM, *et al.* Human multidrug resistance associated protein 4 confers resistance to camptothecins. Pharm Res 2005; 22: 1837-53.

[325] Tian Q, Zhang J, Chan SY, *et al.* Topotecan is a substrate for multidrug resistance associated protein 4. Curr Drug Metab 2006; 7: 105-18.

[326] Brangi M, Litman T, Ciotti M, *et al.* Camptothecin resistance: role of the ATP-binding cassette (ABC), mitoxantrone-resistance half-transporter (MXR), and potential for glucuronidation in MXR-expressing cells. Cancer Res 1999; 59: 5938-46.

[327] Hoki Y, Fujimori A, Pommier Y. Differential cytotoxicity of clinically important camptothecin derivatives in P-glycoprotein-

overexpressing cell lines. Cancer Chemother Pharmacol 1997; 40: 433-8.

[328] Maliepaard M, van Gastelen MA, de Jong LA, *et al.* Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line. Cancer Res 1999; 59: 4559-63.

[329] Gounder MK, Nazar AS, Saleem A, *et al.* Effects of drug efflux proteins and topoisomerase I mutations on the camptothecin analogue gimatecan. Invest New Drugs 2008; 26: 205-13.

[330] Taniguchi K, Wada M, Kohno K, *et al.* A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation. Cancer Res 1996; 56: 4124-9.

[331] Burger H, van Tol H, Boersma AW, *et al.* Imatinib mesylate (STI571) is a substrate for the breast cancer resistance protein (BCRP)/ABCG2 drug pump. Blood 2004; 104: 2940-2.

[332] Houghton PJ, Germain GS, Harwood FC, *et al.* Imatinib mesylate is a potent inhibitor of the ABCG2 (BCRP) transporter and reverses resistance to topotecan and SN-38 *in vitro*. Cancer Res 2004; 64: 2333-7.

[333] Mahon FX, Belloc F, Lagarde V, *et al.* MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. Blood 2003; 101: 2368-73.

[334] Widmer N, Colombo S, Buclin T, Decosterd LA. Functional consequence of MDR1 expression on imatinib intracellular concentrations. Blood 2003; 102: 1142.

[335] Polli JW, Humphreys JE, Harmon KA, *et al.* The role of efflux and uptake transporters in [N-{3-chloro-4-[(3-fluorobenzyl)oxy] phenyl}-6-[5-({[2-(methylsulfonyl)ethy l]amino}methyl)-2-furyl]-4-quinazolinamine (GW572016, lapatinib) disposition and drug interactions. Drug Metab Dispos 2008; 36: 695-701.

[336] Robey RW, Obrzut T, Shukla S, *et al.* Becatecarin (rebeccamycin analog, NSC 655649) is a transport substrate and induces expression of the ATP-binding cassette transporter, ABCG2, in lung carcinoma cells. Cancer Chemother Pharmacol 2009; 64: 575-83.

[337] Robey RW, Medina-Perez WY, Nishiyama K, *et al.* Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells. Clin Cancer Res 2001; 7: 145-52.

[338] Saeki T, Ueda K, Tanigawara Y, Hori R, Komano T. Human P-glycoprotein transports cyclosporin A and FK506. J Biol Chem 1993; 268: 6077-80.

[339] Dahan A, Sabit H, Amidon GL. Multiple efflux pumps are involved in the transepithelial transport of colchicine: combined effect of p-glycoprotein and multidrug resistance-associated protein 2 leads to decreased intestinal absorption throughout the entire small intestine. Drug Metab Dispos 2009; 37: 2028-36.

[340] Callaghan R, Riordan JR. Synthetic and natural opiates interact with P-glycoprotein in multidrug-resistant cells. J Biol Chem 1993; 268: 16059-64.

[341] Tournier N, Chevillard L, Megarbane B, *et al.* Interaction of drugs of abuse and maintenance treatments with human P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2). Int J Neuropsychopharmacol 2010; 13: 905-15.

[342] van de Wetering K, Zelcer N, Kuil A, *et al.* Multidrug resistance proteins 2 and 3 provide alternative routes for hepatic excretion of -glucuronides. Mol Pharmacol 2007; 72: 387-94.

[343] Luna-Tortos C, Fedrowitz M, Loscher W. Several major antiepileptic drugs are substrates for human P-glycoprotein. Neuropharmacology 2008; 55: 1364-75.

[344] Luna-Tortos C, Fedrowitz M, Loscher W. Evaluation of transport of common antiepileptic drugs by human multidrug resistance-associated proteins (MRP1, 2 and 5) that are overexpressed in pharmacoresistant epilepsy. Neuropharmacology 2010; 58: 1019-32.

[345] Cerveny L, Pavek P, Malakova J, Staud F, Fendrich Z. Lack of interactions between breast cancer resistance protein (bcrp/abcg2) and selected antiepileptic agents. Epilepsia 2006; 47: 461-8.

[346] Luna-Tortos C, Rambeck B, Jurgens UH, Loscher W. The Antiepileptic Drug Topiramate is a Substrate for Human P-glycoprotein but Not Multidrug Resistance Proteins. Pharm Res 2009; 26: 2464-70.

[347] Vautier S, Lacomblez L, Chacun H, *et al.* Interactions between the dopamine agonist, bromocriptine and the efflux protein, P-glycoprotein at the blood-brain barrier in the mouse. Eur J Pharm Sci 2006; 27: 167-74.

[348] Uhr M, Ebinger M, Rosenhagen MC, Grauer MT. The anti-Parkinson drug budipine is exported actively out of the brain by P-glycoprotein in mice. Neurosci Lett 2005; 383: 73-6.

[349] Soares-Da-Silva P, Serrao MP. Outward transfer of dopamine precursor L-3,4-dihydroxyphenylalanine (L-dopa) by native and human P-glycoprotein in LLC-PK(1) and LLC-GA5 col300 renal cells. J Pharmacol Exp Ther 2000; 293: 697-704.

[350] El Ela AA, Hartter S, Schmitt U, *et al.* Identification of P-glycoprotein substrates and inhibitors among psychoactive compounds--implications for pharmacokinetics of selected substrates. J Pharm Pharmacol 2004; 56: 967-75.

[351] Boulton DW, DeVane CL, Liston HL, Markowitz JS. *In vitro* P-glycoprotein affinity for atypical and conventional antipsychotics. Life Sci 2002; 71: 163-9.

[352] Uhr M, Grauer MT. abcb1ab P-glycoprotein is involved in the uptake of citalopram and trimipramine into the brain of mice. J Psychiatr Res 2003; 37: 179-85.

[353] Hayeshi R, Masimirembwa C, Mukanganyama S, Ungell AL. The potential inhibitory effect of antiparasitic drugs and natural products on P-glycoprotein mediated efflux. Eur J Pharm Sci 2006; 29: 70-81.

[354] Vezmar M, Georges E. Direct binding of chloroquine to the multidrug resistance protein (MRP): possible role for MRP in chloroquine drug transport and resistance in tumor cells. Biochem Pharmacol 1998; 56: 733-42.

[355] Wu CP, Klokouzas A, Hladky SB, Ambudkar SV, Barrand MA. Interactions of mefloquine with ABC proteins, MRP1 (ABCC1) and MRP4 (ABCC4) that are present in human red cell membranes. Biochem Pharmacol 2005; 70: 500-10.

[356] Dohgu S, Yamauchi A, Takata F, *et al.* Uptake and efflux of quinacrine, a candidate for the treatment of prion diseases, at the blood-brain barrier. Cell Mol Neurobiol 2004; 24: 205-17.

[357] Merino G, Jonker JW, Wagenaar E, *et al.* Transport of anthelmintic benzimidazole drugs by breast cancer resistance protein (BCRP/ABCG2). Drug Metab Dispos 2005; 33: 614-8.

[358] He Y, Liu Y, Zeng S. Stereoselective and multiple carrier-mediated transport of cetirizine across Caco-2 cell monolayers with potential drug interaction. Chirality 2010; 22: 684-92.

[359] Cvetkovic M, Leake B, Fromm MF, Wilkinson GR, Kim RB. OATP and P-glycoprotein transporters mediate the cellular uptake and excretion of fexofenadine. Drug Metab Dispos 1999; 27: 866-71.

[360] Matsushima S, Maeda K, Ishiguro N, Igarashi T, Sugiyama Y. Investigation of the inhibitory effects of various drugs on the hepatic uptake of fexofenadine in humans. Drug Metab Dispos 2008; 36: 663-9.

[361] Hassan HE, Myers AL, Coop A, Eddington ND. Differential involvement of P-glycoprotein (ABCB1) in permeability, tissue distribution, and antinociceptive activity of methadone, buprenorphine, and diprenorphine: *in vitro* and *in vivo* evaluation. J Pharm Sci 2009; 98: 4928-40.

[362] Takara K, Tsujimoto M, Ohnishi N, Yokoyama T. Effects of continuous exposure to digoxin on MDR1 function and expression in Caco-2 cells. J Pharm Pharmacol 2003; 55: 675-81.

[363] Haslam IS, Jones K, Coleman T, Simmons NL. Induction of P-glycoprotein expression and function in human intestinal epithelial cells (T84). Biochem Pharmacol 2008; 76: 850-61.

[364] Narang VS, Fraga C, Kumar N, *et al.* Dexamethasone increases expression and activity of multidrug resistance transporters at the rat blood-brain barrier. Am J Physiol Cell Physiol 2008; 295: C440-C450.

[365] Demeule M, Jodoin J, Beaulieu E, Brossard M, Beliveau R. Dexamethasone modulation of multidrug transporters in normal tissues. FEBS Lett 1999; 442: 208-14.

[366] Schrenk D, Baus PR, Ermel N, *et al.* Up-regulation of transporters of the MRP family by drugs and toxins. Toxicol Lett 2001; 120: 51-7.

[367] Huwyler J, Wright MB, Gutmann H, Drewe J. Induction of cytochrome P450 3A4 and P-glycoprotein by the isoxazolyl-penicillin antibiotic flucloxacillin. Curr Drug Metab 2006; 7: 119-26.

[368] Notenboom S, Wouterse AC, Peters B, *et al.* Increased apical insertion of the multidrug resistance protein 2 (MRP2/ABCC2) in renal proximal tubules following gentamicin exposure. J Pharmacol Exp Ther 2006; 318: 1194-202.

[369] Huang R, Murry DJ, Kolwankar D, Hall SD, Foster DR. Vincristine transcriptional regulation of efflux drug transporters in carcinoma cell lines. Biochem Pharmacol 2006; 71: 1695-704.

[370] Jigorel E, Le Vee M, Boursier-Neyret C, Parmentier Y, Fardel O. Differential regulation of sinusoidal and canalicular hepatic drug transporter expression by xenobiotics activating drug-sensing receptors in primary human hepatocytes. Drug Metab Dispos 2006; 34: 1756-63.

[371] Magnarin M, Morelli M, Rosati A, *et al.* Induction of proteins involved in multidrug resistance (P-glycoprotein, MRP1, MRP2, LRP) and of CYP 3A4 by rifampicin in LLC-PK1 cells. Eur J Pharmacol 2004; 483: 19-28.

[372] Harmsen S, Meijerman I, Febus CL, *et al.* PXR-mediated induction of P-glycoprotein by anticancer drugs in a human colon adenocarcinoma-derived cell line. Cancer Chemother Pharmacol 2010; 66: 765-71.

[373] Fardel O, Lecureur V, Daval S, Corlu A, Guillouzo A. Up-regulation of P-glycoprotein expression in rat liver cells by acute doxorubicin treatment. Eur J Biochem 1997; 246: 186-92.

[374] Yoshida M, Suzuki T, Komiya T, *et al.* Induction of MRP5 and SMRP mRNA by adriamycin exposure and its overexpression in human lung cancer cells resistant to adriamycin. Int J Cancer 2001; 94: 432-7.

[375] Ross DD, Yang W, Abruzzo LV, *et al.* Atypical multidrug resistance: breast cancer resistance protein messenger RNA expression in mitoxantrone-selected cell lines. J Natl Cancer Inst 1999; 91: 429-33.

[376] Nieth C, Lage H. Induction of the ABC-transporters Mdr1/P-gp (Abcb1), mrpl (Abcc1), and bcrp (Abcg2) during establishment of multidrug resistance following exposure to mitoxantrone. J Chemother 2005; 17: 215-23.

[377] Takara K, Tsujimoto M, Kokufu M, Ohnishi N, Yokoyama T. Up-regulation of MDR1 function and expression by cisplatin in LLC-PK1 cells. Biol Pharm Bull 2003; 26: 205-9.

[378] Kauffmann HM, Keppler D, Kartenbeck J, Schrenk D. Induction of cMrp/cMoat gene expression by cisplatin, 2-acetylaminofluorene, or cycloheximide in rat hepatocytes. Hepatology 1997; 26: 980-5.

[379] Oguri T, Isobe T, Suzuki T, *et al.* Increased expression of the MRP5 gene is associated with exposure to platinum drugs in lung cancer. Int J Cancer 2000; 86: 95-100.

[380] Burger H, van Tol H, Brok M, *et al.* Chronic imatinib mesylate exposure leads to reduced intracellular drug accumulation by induction of the ABCG2 (BCRP) and ABCB1 (MDR1) drug transport pumps. Cancer Biol Ther 2005; 4: 747-52.

[381] Kauffmann HM, Keppler D, Gant TW, Schrenk D. Induction of hepatic mrp2 (cmrp/cmoat) gene expression in nonhuman primates treated with rifampicin or tamoxifen. Arch Toxicol 1998; 72: 763-8.

[382] Aquilante CL, Letrent SP, Pollack GM, Brouwer KL. Increased brain P-glycoprotein in morphine tolerant rats. Life Sci 2000; 66: L47-L51.

[383] Yousif S, Saubamea B, Cisternino S, *et al.* Effect of chronic exposure to morphine on the rat blood-brain barrier: focus on the P-glycoprotein. J Neurochem 2008; 107: 647-57.

[384] Furuya KN, Thottassery JV, Schuetz EG, Sharif M, Schuetz JD. Bromocriptine transcriptionally activates the multidrug resistance gene (pgp2/mdr1b) by a novel pathway. J Biol Chem 1997; 272: 11518-25.

[385] Burk O, Arnold KA, Nussler AK, *et al.* Antimalarial artemisinin drugs induce cytochrome P450 and MDR1 expression by activation of xenosensors pregnane X receptor and constitutive androstane receptor. Mol Pharmacol 2005; 67: 1954-65.

Received: February 11, 2010 Revised: April 06, 2010 Accepted: April 06, 2010

Improving Cancer Chemotherapy with Modulators of ABC Drug Transporters

S. Shukla, S. Ohnuma and S. V. Ambudkar*

Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland 20892, USA

Abstract: ATP-binding cassette (ABC) transporters, P-glycoprotein (P-gp, ABCB1) and ABCG2, are membrane proteins that couple the energy derived from ATP hydrolysis to efflux many chemically diverse compounds across the plasma membrane, thereby playing a critical and important physiological role in protecting cells from xenobiotics. These transporters are also implicated in the development of multidrug resistance (MDR) in cancer cells that have been treated with chemotherapeutics. One approach to blocking the efflux capability of an ABC transporter in a cell or tissue is inhibiting the activity of the transporters with a modulator. Since ABC transporter modulators can be used in combination with chemotherapeutics to increase the effective intracellular concentration of anticancer drugs, the possible impact of modulators of ABC drug transporters is of great clinical interest. Another possible clinical use of modulators that has recently attracted attention is their ability to increase oral bioavailability or increase tissue penetration of drugs transported by the transporters. Several preclinical and clinical studies have been performed to evaluate the feasibility and the safety of this approach. The primary focus of this review is to discuss progress made in recent years in the identification and applicability of compounds that may serve as ABC transporter modulators and the possible role of these compounds in altering the pharmacokinetics and pharmacodynamics of therapeutic drugs used in the clinic.

Keywords: ABC transporters, ABCG2, blood-brain barrier, chemotherapy, modulators, multidrug resistance, oral bioavailability, P-glycoprotein.

MULTIDRUG RESISTANCE AND ABC DRUG TRANSPORTERS

Chemotherapy is usually the most effective treatment for cancer patients with advanced/metastatic tumors or hematological malignancies. However, cancer cells often develop simultaneous resistance to many functionally and structurally unrelated anti-cancer drugs, a phenomenon known as multidrug resistance (MDR), which is a major problem in the treatment of cancer [1]. Cancer cells with the MDR phenotype may have either inherent resistance to anti-cancer drugs or resistance acquired after cycles of chemotherapy. Several mechanisms of anti-cancer drug resistance, such as reduced cellular drug uptake, increased or decreased expression of metabolic enzymes, mutation of the target, alterations of the apoptotic pathway, changes in cellular repair mechanisms and increased expression/activity of drug efflux pumps have been characterized [2].

One of the most important MDR mechanisms is an increased efflux rate of the anti-cancer drug from cancer cells by the members of the superfamily of ATP-binding cassette (ABC) transporters, which is one of the largest protein families in living organisms [3, 4]. There are 48 genes in the human genome that encode ABC transporters, which are divided into seven subfamilies (ABCA-ABCG) based on the amino acid sequence identity of ATP-binding domains [4]. ATP-binding domains, also known as nucleotide binding domains (NBDs), and trans-membrane domains (TMDs), each with six alpha helices, are the essential units of an ABC transporter. The NBD has the highly conserved Walker A, B, and signature C motifs and are well conserved in all the organisms. On the other hand, the TMD confers transport substrate specificity, and is not well conserved [5, 6]. The members of this family play a crucial role in physiology, and mutations in ABC transporters result in several human diseases including progressive familial intrahepatic cholestasis (ABCB11), Dubin-Johnson syndrome (ABCC2), cystic fibrosis (ABCC7), and adrenoleukodystrophy (ABCD1) [3, 4]. So far, at least 15 transporters have been shown to export anti-cancer drugs *in vitro* [7]. Among them, P-glycoprotein (P-gp; MDR1, ABCB1), multidrug resistance-associated protein 1 (MRP1, ABCC1), and ABCG2 (breast cancer resistance protein; BCRP, mitoxantrone resistance protein; MXR) are considered major players in the development of MDR in cancer cells.

P-gp, discovered in 1976, is one of the best characterized ABC transporters [8]. It is composed of two homologous halves, each containing a NBD and a TMD, and transports exogenous and endogenous amphipathic substrates out of cells using energy from ATP [9]. It is localized at the apical surface of the cells and is highly expressed in capillary endothelial cells of the blood-brain barrier, placental trophoblasts, the testes, intestines, the liver, kidneys and the adrenal gland [3]. These tissues function as barriers, suggesting the physiological role of P-gp is to protect the body from xenobiotics and toxins. P-gp pumps out many structurally unrelated anti-cancer drugs, such as vinca alkaloids (vinblastine, vincristine, vindesine, vinorelbine), anthracyc-

*Address correspondence to this author at the Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland 20892, USA; Tel: 301-402-4178; Fax: 301-435-8188;
E-mail: ambudkar@helix.nih.gov

1389-4501/11 $58.00+.00 © 2011 Bentham Science Publishers Ltd.

lines (doxorubicin, daunorubicin) and taxanes (paclitaxel, docetaxel), suggesting the flexible nature of the substrate binding site of P-gp [10, 11]. P-gp is highly expressed in leukemia, breast, ovarian, colon, kidney, adrenocortical, and hepatocellular cancers and its overexpression is inversely correlated with poor clinical prognosis [12-14].

ABCG2 is a half transporter which contains one TMD and one NBD, and is therefore thought to homodimerize or oligomerize to form the functional unit [15-17]. Interestingly, similar to the MDR family of transporters in yeast, the location of the TMD and NBD is reversed in ABCG2 compared to P-gp [18]. Similar to P-gp, ABCG2 is localized to the apical membrane in epithelial cells and normally expressed in organs such as the placenta, brain, liver, prostate, and intestine [16]. ABCG2 is also detected in hematopoietic and other stem cells, suggesting that it may play an important role in the protective function of pluripotent stem cells [19]. Overexpression of ABCG2 renders cancer cells resistant to many anti-cancer drugs including mitoxantrone, topotecan and methotrexate and it is associated with poor response to chemotherapy in leukemia and breast cancer patients [20, 21].

MRP1 (ABCC1) was the first member of the MRP family to be identified (in 1992) and has been linked to the development of MDR [22]. The structure of MRP1 is similar to that of P-gp, except five additional transmembrane helices are present at the amino-terminal end of the transporter. It is highly expressed in the adrenal gland, bladder, choroid plexus, colon, in erythrocytes, bone marrow, the kidneys, lungs, placenta, spleen, stomach, testes, in helper T cells and in muscle cells [23]. MRP1 transports some substrates conjugated with glucuronide, sulfate or glutathione, vinca alkaloids, anthracyclines, methotrexate and also leukotriene C4, which is an endogenous substrate for the transporter [24, 25]. The localization of MRP1 is different from that of P-gp, as it is expressed in the basolateral membrane in polarized epithelial cells and transports substrates in to the bloodstream [26]. Overexpression of MRP1 has also been shown in lung, breast, prostate, and ovarian cancer, gastrointestinal carcinoma, melanoma, and leukemia [27]. While some studies have reported MRP1 expression levels to be of prognostic significance [28, 29], others have found no correlation between clinical outcome and its expression [30, 31]. A comprehensive role of MRP1 in clinical drug resistance is still debatable; therefore the present review will mainly focus on two major ABC drug transporters, P-gp and ABCG2.

APPROACHES TO IMPROVING CHEMOTHERAPY

A combination of two or multiple drugs is often used in chemotherapy, as each drug inhibits a specific target and the combination therefore could maximize the killing effect on cancer cells, additively and synergistically [32]. The combination of drugs targets several cellular pathways simultaneously, which not only augments the tumoricidal effect of anti-cancer drugs, but also lessens the occurrence of drug resistance, thereby providing an optimal therapeutic outcome [2].

In addition to conventional anti-cancer drugs, many novel drugs have been developed based on an understanding of molecular mechanisms, which revealed a number of new potential targets for drugs. These novel agents such as the monoclonal antibody cetuximab, which targets the extracellular domain of EGFR by blocking ligand binding, and the small-molecule inhibitor erlotinib, that blocks EGFR intracellular tyrosine kinase activity, have demonstrated clinical advantages over the established drug regimen [33]. Several newer approaches such as cancer vaccines, antisense oligonucleotides, and small-interfering RNAs (siRNAs) are also promising approaches for targeting cancer cells and deserve further clinical investigation [33]. These approaches show a great promise for improving the efficiency of chemotherapy, but are still not sufficient to alter the outcome for many cancer patients.

ABC DRUG TRANSPORTERS: TARGETS FOR IMPROVING CHEMOTHERAPY

MDR mediated by ABC transporters in cancer cells can be overcome by methods such as specific inhibition of transporters at the cell surface level, blocking the signaling pathways that control amplification and overexpression of these transporters, and targeting the transcription factors that regulate the expression of the pumps [34]. Historically, research on the reversal of ABC transporter-mediated MDR has been directed towards inhibiting the activity of the transporter at the cell surface by drugs known as modulators or inhibitors and due to the scope of this review, we will focus on this strategy as a possible way to improve chemotherapy and/or oral bioavailability. A majority of modulator drugs used for this purpose are basically transport substrates of ABC drug transporters that inhibit efflux of anti-cancer agents in both *in vitro* and *in vivo* systems.

Almost three decades ago, Tsuruo *et al.* discovered that verapamil (a calcium channel blocker used as a coronary vasodilator) enhanced the cytotoxicity of vincristine and vinblastine in a P-gp-expressing vincristine-resistant cell line [35]. Slater *et al.* later reported that cyclosporine A (CsA), an immunosuppressant drug, completely reversed primary resistance to vincristine and daunorubicin in a drug-resistant cell line of human T cell acute lymphatic leukemia [36]. Additional studies showed that both verapamil and CsA reversed the MDR phenotype by interacting directly at the drug-substrate site on P-gp [37-39]. Several other 'off the shelf' drugs which were already in clinical use such as nicardipine and nifedipine were shown to reverse Pgp-mediated MDR (reviewed in [34]) but many of these agents were toxic, as higher concentrations of these drugs were required for inhibiting the transporters. These drugs have been described as the first generation modulators of P-gp. In 1991, an analog of CsA PSC833 (valspodar) was synthesized which was not only 10-fold more potent than CsA in inhibiting Pgp activity but also showed no immunosuppressive side effect [40]. Discovery of this molecule marked the beginning of the second generation of ABC transporter modulators. Many other modulators were developed based on the quantitative structural activity relationship approach. A number of clinical studies were carried out using these modulators. These trials demonstrated some advantage over the previous agents, but patients still suffered from side effects associated with the therapy [41-44]. Since then, third generation modulators such as tariquidar (XR9576) [45],

elacridar (GF120918) [46], zosuquidar (LY335979) [47] and dofequidar [48] have been developed which have shown improved selectivity and inhibitory activity towards ABC transporters. Martin *et al*. reported that tariquidar, unlike verapamil and CsA, was not a transport substrate of P-gp, and that it inhibits P-gp function by binding at a site distinct from the vinblastine and paclitaxel site [49]. Tariquidar potentiated the cytotoxicity of several drugs including doxorubicin, paclitaxel, etoposide, and vincristine at very low concentrations (25-80 nM). Co-administration of tariquidar potentiated the antitumor activity of doxorubicin without a significant increase in toxicity in mice [50]. Two Phase III trials of tariquidar in combination with paclitaxel/ carboplatin, or vinorelbine were initiated in patients with non-small-cell lung cancer (NSCLC) but these studies were terminated later owing to chemotherapy-related toxicity in the tariquidar arm [51]. Recent results of another phase I study with vinorelbine have indicated shown that tariquidar is a potent P-gp antagonist, without significant side effects and much less pharmacokinetic interaction than previous P-gp antagonists [52]. This and other ongoing clinical studies at the National Cancer Institute (NIH, Bethesda, USA) with tariquidar suggest that there is still optimism that therapeutic modulation of MDR mediated by ABC transporters may be beneficial to patients.

The leukotriene LTD_4 receptor antagonist MK571 and a fungal toxin, fumitremorgin C (FTC) were identified as specific modulators of MRP1 and ABCG2, respectively [53, 54]. Since then MK571 and FTC have been used as standard modulators for inhibiting MRP1- and ABCG2-mediated transport, respectively, in laboratory assays. However, unlike P-gp, clinically useful modulators of MRP1 and ABCG2 have not been extensively investigated in clinical trials. Some modulators of P-gp such as biricodar (VX-710) dofequidar (MS-209), elacridar (GF120918) or CBT-1 have also been shown to inhibit MRP-1 and/or ABCG2-mediated drug transport/resistance in *in vitro* and in clinical studies [48, 55-61]. Although the effect of these modulators on each transporter is not well established in clinical trials, these drugs might be useful against cancer cells which express a combination of transporters linked to MDR. The efficacy and safety of these modulators in clinical studies is still under evaluation.

Newer approaches have been developed in the laboratory to overcome ABC transporter-mediated MDR, including a monoclonal antibody that binds to human P-gp and inhibits drug transport [62], siRNA technology that specifically decreases the expression of *ABCB*1 [63], and regulation of transcription factors that inhibit P-gp activation [64]. Although these approaches might help to overcome transporter-mediated MDR, clinical studies using these alternative approaches are still lacking to establish their efficacy in cancer patients.

MODULATORS OF ABC DRUG TRANSPORTERS AND ORAL BIOAVAILABILITY OF CHEMOTHERAPEUTICS

Although modulators of ABC drug transporters were initially envisioned as drugs that can be used to overcome ABC transporter-mediated MDR in cancer cells or tumor tissue, these drugs are recapturing attention for another possible use: increasing oral bioavailability of drugs which were earlier ruled out as oral chemotherapeutic agents due to poor bioavailability [65]. Pgp and ABCG2 have now been confirmed to play significant physiological roles including preventing the uptake of many drugs and food components from the gut into the body. Both P-gp and ABCG2 are expressed in the brush-border membranes of enterocytes in the intestine, which results in the excretion of their drug-substrates into the lumen, resulting in a potentially limiting net absorption (Fig. **1**). The association of drug transporter levels in the intestine and oral drug bioavailability was

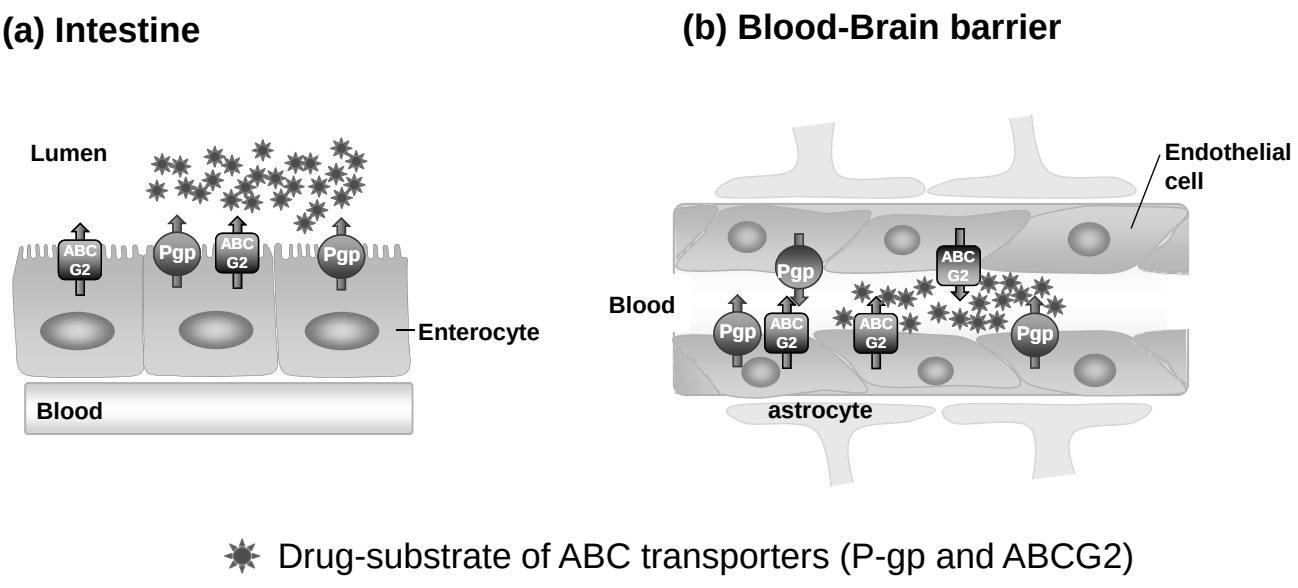


Fig. (1). Schematic showing the localization of P-gp and ABCG2 at the apical surface in the (**a**) intestinal enterocytes and **b**) brain capillary endothelial cells. The drug substrates of the transporters are pumped inside the lumen of the intestine and brain capillaries by these transporters, resulting in reduced oral bioavailability and decreased brain accumulation (see text for details).

Table 1. Preclinical/Clinical Studies Demonstrating the Use of Modulators of ABC Drug Transporters to Improve Oral Bioavailability

Transporter	Drug	Inhibitor	References
P-gp	Paclitaxel	HM30181	[69]
P-gp	Paclitaxel	Valspodar (PSC833)	[93]
P-gp	Docetaxel	Ritonavir	[84]
P-gp	Talinolol	Verapamil	[94]
P-gp	Paclitaxel, Digoxin, Fexofenadine	Biochanin	[95]
P-gp	Paclitaxel	Elacridar (GF120918)	[96]
P-gp	Paclitaxel	Cyclosporin A	[97]
P-gp	Docetaxel	Ritonavir	[98]
P-gp	Paclitaxel	Cyclosporin A	[99]
P-gp	Digoxin	Quinidine	[100]
P-gp	Docetaxel	Cyclosporin A	[101]
P-gp	Digoxin	Talinolol	[102]
P-gp	Paclitaxel	Elacridar (GF120918)	[103]
P-gp	Paclitaxel	HM30181	[69]
P-gp, ABCG2	Topotecan	Elacridar (GF120918)	[82, 104]
P-gp, ABCG2	Etoposide	Elacridar (GF120918)	[105]
P-gp, ABCG2	Topotecan	Elacridar (GF120918)	[106]
ABCG2	Sulfasalazine	Curcumin	[68]
ABCG2	Methotrexate	Pantoprazole	[107]
ABCG2	Irinotecan	Gefitinib	[108]
ABCG2	Methotrexate	Omeprazole/lansoprazole	[109]

Oral bioavailability of Erlotinib [110], Gefitinib [111] and Paclitaxel [112] has been shown to be increased in *mdr1a, mdr1b, Abcg2* triple knock-out and *mdr1a, mdr1b* knock-out mice, respectively.

initially demonstrated by Spareboom *et al.*, who showed that P-gp in the intestine limits the uptake of orally administered paclitaxel [66]. Later Greiner *et al.* also showed that the plasma concentration of digoxin, another P-gp substrate, was inversely correlated with intestinal P-gp expression in patients [67]. These observations led researchers to evaluate if modulators of ABC transporters could possibly be used to temporarily inhibit the efflux activities of the transporters, thereby increasing the oral bioavailability and plasma concentrations of some poorly absorbed drugs. Several preclinical *in vitro* and *in vivo* studies have since then reported the use of specific modulators of ABC drug transporters to increase the oral bioavailability or efficacy of drugs such as docetaxel, vinblastine, etoposide, digoxin, indinavir, saquinavir, tacrolimus, nelfinavir, talinolol, topotecan, methotrexate, irinotecan, SN-38, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and erlotinib, which are either P-gp or ABCG2 substrates (reviewed in [65]). This is consistent with biochemical studies demonstrating that there is significant overlap in substrate specificity of Pgp, ABCG2 and MRP1 [11]. Recently, we showed that inhibition of intestinal abcg2 in mice by curcumin results in increased oral bioavailability of its substrate, sulfalasalazine, which suggested that curcumin, a non-toxic natural product modulator of P-gp and ABCG2, may be used to enhance drug exposure [68]. A very recent study reported a newly discovered modulator of Pgp, HM30181 increased oral bioavailability of paclitaxel in rats and inhibition of growth of tumor xenografts in mice which was better than intravenous paclitaxel [69]. These results identify HM30181, as a highly selective and potent inhibitor of MDR1, which in combination with paclitaxel may provide an orally effective anti-tumor regimen. Table **1** summarizes recent studies which reported the use of P-gp or ABCG2 modulators to increase the oral bioavailability or plasma concentrations of drugs. These studies suggest a pharmacological advantage of using chemotherapeutic drugs in combination with modulators of ABC drug transporters.

Although using modulators in combination with other drugs is an appealing strategy, it does have the risk of increased toxicities associated with the combination therapy because both P-gp and ABCG2 are also expressed in epithelial cells of the colon, adrenal cortex, kidney, liver and bile canalicular membranes and gall bladder. These organs influence the excretion of drugs. Moreover, as P-gp and ABCG2 also protect vital organs such as the brain, the testes, and the fetus, against toxins that enter the body, inhibition of the activity of these transporters may lead to toxicity associated with these organs. In addition, some modulators also inhibit metabolic enzymes such as cytochrome P450-3A4, which may significantly delay drug clearance from the

Table 2. Preclinical/Clinical Studies Demonstrating Use of Modulators of ABC Drug Transporters to Improve Brain Accumulation of Drugs

Transporter	Drug	Inhibitor	References
P-gp	Vincristine	Quercetin	[113]
P-gp	Colchicine, Vinblastine	Valspodar (PSC833), Elacridar (GF120918)	[114]
P-gp	Colchicine, Vinblastine	Verapamil, Valspodar (PSC-833)	[115, 116]
P-gp	Colchicine	Valspodar (PSC 833)	[117]
P-gp	Paclitaxel	Cyclosporin A, Valspodar (PSC833), Elacridar (GF120918)	[118]
P-gp	Paclitaxel	Zosuquidar (LY335979)	[119]
P-gp	Paclitaxel	PSC833 (Valspodar)	[120]
P-gp, ABCG2	Dasatinib	Elacridar (GF120918), Zosuquidar (LY335979) (only for P-gp)	[121]
P-gp, ABCG2	Dasatinib	Elacridar (GF120918)	[78]
P-gp	Imatinib	Valsopodar (PSC 833), Zosuquidar (LY335979)	[80, 122]
P-gp, ABCG2	Imatinib	Elacridar (GF120918), Pantoprazole	[79]
ABCG2	Imatinib	Elacridar (GF120918)	[80, 122]
ABCG2	Mitoxantrone	Elacridar (GF120918)	[123]
ABCG2	Mitoxantrone	Elacridar (GF120918)	[124]

Brain accumulation of Topotecan [77] and Dasatinib [121] has been shown to be increased in *mdr1a, mdr1b, Abcg2* triple knock-out mice.

body [70]. Considering these factors, the use of modulators in a combination regimen for improving oral bioavailability is an approach which should be carefully monitored for minimal toxicity associated with improved therapeutic outcome.

MODULATORS OF ABC DRUG TRANSPORTERS INCREASE BRAIN ACCUMULATION OF CHEMOTHERAPEUTICS

P-gp and ABCG2 expression in the brain has been found in numerous species, including humans, primates, rats, mice, and pigs [71]. These transporters are principally expressed at the luminal membrane of the endothelial cells in brain capillaries (blood-brain barrier; BBB) and pump substrates back into the circulation, which is a critical determinant for the efficacy/toxicity of chemotherapeutics for the brain (Fig. **1**). The importance of P-gp in the brain was first demonstrated by Schinkel *et al*., who showed that brain uptake of drugs was higher in P-gp knockout mice, suggesting that Pgp protects the brain from toxic effects under physiological conditions [72]. Later, several other studies reported that P-gp drug-substrate levels were significantly higher in the brains of mdr1a knockout mice than wild-type mice [73-76]. Brain accumulation of topotecan, a camptothecin analog and a substrate of both P-gp and ABCG2 that is used for treating ovarian, cervical and small cell lung cancer, was also found to be 3.7-fold higher in mdr1a/b/Abcg2-knockout mice compared to wild-type mice [77]. Recently, Lagas *et al.* also showed P-gp and ABCG2 knockout mice have drastically increased dasatinib brain concentrations, both after oral and i.p. administration [78]. Collectively, these studies provide strong evidence for the notion that inhibition of ABC transporters at the BBB can also be used to enhance the efficacy of drugs which are targeted for brain disorders and do not penetrate the BBB by virtue of being substrates of the transporters. This has been demonstrated in several *in vivo* and *in vitro* studies in which modulators of ABC transporters such as elacridar, pantoprazole, valspodar and zosuquidar were used to increase accessibility of several clinically important drugs into the brain [78-80]. Table **2** summarizes the list of modulators that have been evaluated for the purpose of increasing brain accumulation of clinically important drugs. Taken together, modulators of ABC drug transporters can possibly be used to augment brain accumulation of drugs which cannot cross the BBB due to the action of transporters, thereby increasing their therapeutic potential.

MODULATORS AS ADJUVANTS IN CLINICAL STUDIES

As stated above, inhibition of ABC drug transporters by modulators may be used to achieve two independent clinical objectives: (1) reversal of MDR in drug resistant tumors during chemotherapy (2) increasing a drug's exposure in body {tissues} for purposes such as increasing oral bioavailability or enhanced brain accumulation of chemotherapeutics. Inhibiting ABC transporters as a way to reverse drug resistance in tumors has been reported several times, but most studies failed to show a dramatic improvement in clinical outcome. We have earlier reviewed the recent progress made in clinical studies by third generation ABC transporter modulators [34]. Here, we will describe recent clinical studies reporting promising results. In a Phase I/II clinical trial, Zosuquidar (LY335979) in combination with CHOP (cyclophosphamide, hydroxydaunorubicin, oncovin (vincristine), and prednisone) was shown to have little effect on the pharmacokinetics of anti-cancer drugs in patients with non-Hodgkin's lymphoma, which suggests that this combination may be pursued further in phase III studies [81].

Another phase I dose escalation study showed that a combination of oral topotecan and tariquidar was well tolerated in patients and warranted a further phase II evaluation [82]. Saeki *et al.* also reported that a dual inhibitor of Pgp and MRP1, dofequidar (MS-209) in combination with cyclophosphamide, doxorubicin, and fluorouracil (CAF) displayed a significantly increased efficacy in breast cancer patients who did not receive prior therapy. Although the patient numbers in these analyses were small, the results are important within these clinically significant patient populations [83]. In addition, studies from Oostendorp *et al.* also suggest that co-administration of ritonavir with docetaxel could also be evaluated for its efficacy in patients with solid tumors [84]. Although more positive outcomes are expected now because of the increasing knowledge about modulators with drug transporters, studies such as those reported by Lhomme *et al*. compared the safety and efficacy of paclitaxel administered with or without PSC833, in patients with advanced ovarian or primary peritoneal cancer, showed that the addition of PSC833 to paclitaxel did not lead to an arrest of the disease progression or overall survival and the combination was more toxic compared with paclitaxel in untreated patients with advanced ovarian or primary peritoneal cancer [85].

The apparent lack of response to modulators to reverse MDR in clinical studies can be attributed to multiple factors. One of the most challenging issues in using modulators with chemotherapeutic agents is alteration of the pharmacokinetics of the chemotherapeutic agent, which has a toxic effect. In addition, inhibition of ABC drug transporters adds to non-specific dose limiting toxic effects and it may also result in drug-drug interaction related side effects. As a result, many of these studies had to be discontinued because of toxicities in patients [51]. Genetic variability (SNPs) in ABC transporters (discussed below) and the variability in the expression levels of transporters among individuals selected for the clinical studies may also be one of the factors responsible for the poor outcome of the use of modulators. Another important aspect is patient selection in clinical studies. As development of MDR is a multifactorial phenomenon, reversal of MDR may have been studied in patients in whom drug resistance was not due to the overexpression of ABC drug transporters. Considering this, patients who show higher levels of P-gp or ABCG2 in tumor tissue should be the ones selected to evaluate the modulators in clinical studies. Moreover, the presence of other ABC transporters in tumors can complicate the use of specific modulators of ABC transporters. Taking the above factors into consideration, the use of modulators to inhibit ABC drug transporter mediated-MDR in patients is a strategy that is still a viable approach for treating drug resistant cancer. However, it is critical to find the right inhibitor for the right transporter with the right dosing, which would allow minimal pharmacokinetic interaction of the chemotherapeutic drug and maximal specific inhibition of the transporters in the target tumor.

SINGLE-NUCLEOTIDE POLYMORPHISMS IN ABC DRUG TRANSPORTERS AND ITS ROLE IN MODULATOR EFFICIENCY

ABC drug transporters directly influence drug efficacy and toxicity and the expression of these transporters determines the degree of resistance of cancer cells to chemotherapy. Several single nucleotide polymorphisms (SNPs) in the coding region of both P-gp and ABCG2 have been reported that influence their substrate specificity and interaction with the transporters [reviewed in [86, 87]]. Such genetic variability in transporters often explains the inter-individual variability in interactions with substrate or modulators and drug disposition, ultimately resulting in differences in clinical endpoints including toxicity and response to the modulators. Kimchi-Sarfaty *et al*. reported that a synonymous mutation (C3435T) in a particular MDR1 haplotype causes a change in the conformation of substrate/modulator binding sites [88]. Another example of a common non-synonymous SNP (421C>A) in ABCG2 has been linked to increased oral bioavailability of topotecan in patients, decreased survival of prostate cancer patients and increased exposure to atorvastatin and rosuvastatin, statins commonly used in the treatment of high cholesterol [89-91]. In addition, SNPs in metabolic enzymes such as cytochrome P450s (CYPs) are often found to be associated with altered pharmacokinetics of transporter substrate drugs [92]. This is especially important when the metabolism of the drug/modulator by the CYPs may be the rate-limiting step in drug clearance. Since the efficacy and toxicity of drugs is ultimately determined by plasma pharmacokinetic parameters, studies investigating polymorphisms in ABC drug transporters as they relate to drug administration are becoming increasingly important in the clinical setting. In conclusion, polymorphisms in ABC drug transporters in various ethnic populations may significantly affect the pharmacokinetics of substrates or modulators, suggesting that they may play a more important role than expected in the efficiency of therapeutic treatment of cancer patients.

CONCLUSIONS AND THERAPEUTIC PERSPECTIVE

In the last decade, a huge amount of effort has been invested in the field of ABC drug transporters to identify, develop, and clinically evaluate a variety of agents known to antagonize the function of these transporters as a means of overcoming tumor resistance. The application of this approach—using modulators as adjuvants in chemotherapy especially for drug-resistant tumors is still debatable due to very little success in the clinical studies that have been performed. The major reasons for the failure of this strategy could be explained in retrospect by multiple factors and variable components that are involved in the development of drug resistance in patients. It still seems that it will take some time to develop an ideal modulator that can be used to overcome drug resistance that develops as a result of cancer treatment or one that can be used in combination with other treatments because of complex substrate recognition factors and the physiological relevance of ABC drug transporters. Moreover, these transporters are only one part of a multi-component system that works to develop the drug resistance phenotype in a cancer cell. Physiologically, transporters play a vital role in protecting the cells from xenobiotics. Altering the function of any of these transporters may lead to a severe physiological imbalance resulting in high levels of toxicity. Therefore it is essential to evaluate the following factors critically to expect a successful outcome from the use of

modulators of ABC drug transporters in the clinic: (a) Toxicity is one of the significant issues reported in several clinical studies, as most of these modulators showed non-specific toxic effects which are not considered acceptable during chemotherapy and prevents their safe usage during treatment; (b) Overlapping substrate specificity and redundancy in the expression of ABC transporters for the same substrate makes it very difficult to target one modulator for a specific drug transporter. Therefore, an ideal modulator should be able to specifically recognize and inhibit the ABC drug transporter responsible for drug resistance in a specific tumor; (c) Patient selection for clinical studies is a factor which seems to have been completely ignored in previous clinical studies and may well be a major reason for the failure of those trials. Patients whose tumors express high levels of transporters will obviously receive the most benefit from modulators. Therefore, drug-resistance reversal trials should ideally be performed in individuals with tumors that initially are chemosensitive but develop drug resistance following initial therapy, which is marked by an increase in the expression of ABC drug transporters; (d) In addition, SNPs in an ABC drug transporter are also linked to sensitivity to drugs. Therefore it is imperative to determine polymorphisms in these transporters in patients before making decisions about combination chemotherapy; (e) Improved *in vivo* imaging studies with probes specific for ABC transporters and high throughput genotype analysis can provide a quantitative assessment of the functioning of the transporters and would help in identifying patients for whom modulators of ABC drug transporter may be used as adjuvants to improve the clinical outcome of chemotherapy. It would also be important to evaluate whether the use of modulators of ABC drug transporters would prevent the selection of resistant cells with characteristics of cancer stem cells with increased expression of ABC transporters such as P-gp or ABCG2; (f) It may be worthwhile to test whether short-term treatment with modulators to block P-gp or ABCG2 just prior to chemotherapy would be effective. This type of short-term treatment could minimize not only the toxic side effects associated with the use of modulators but also help to lower the required doses of chemotherapeutic drugs.

ACKNOWLEDGEMENTS

We thank George Leiman for editorial assistance in preparation of the manuscript.

GRANT SUPPORT

This work was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

DECLARATION OF INTEREST

All authors disclose that they do not have any affiliation with any organization with a financial interest, direct or indirect, in the subject matter or materials discussed in the manuscript that may affect the conduct or reporting of the work submitted under the heading.

REFERENCES

[1] Gottesman MM, Ling V. The molecular basis of multidrug resistance in cancer: the early years of P-glycoprotein research. FEBS Lett 2006; 580: 998-1009.

[2] O'Connor R. A review of mechanisms of circumvention and modulation of chemotherapeutic drug resistance. Curr Cancer Drug Targets 2009; 9: 273-80.

[3] Gottesman MM, Ambudkar SV. Overview: ABC transporters and human disease. J Bioenerg Biomembr 2001; 33: 453-8.

[4] Dean M, Annilo T. Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates. Annu Rev Genom Hum Genet 2005; 6: 123-42.

[5] Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol 1999; 39: 361-98.

[6] Sauna ZE, Smith MM, Muller M, Kerr KM, Ambudkar SV. The mechanism of action of multidrug-resistance-linked P-glycoprotein. J Bioenerg Biomembr 2001; 33: 481-91.

[7] Schinkel AH, Jonker JW. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Deliv Rev 2003; 55: 3-29.

[8] Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochimica et Biophysica Acta Biomembr 1976; 455: 152-62.

[9] Sauna ZE, Kim IW, Ambudkar SV. Genomics and the mechanism of P-glycoprotein (ABCB1). J Bioenerg Biomembr 2007; 39: 481-7.

[10] Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2002; 2: 48-58.

[11] Sauna ZE, Ambudkar SV. Evolution and function of the multidrug resistance-linked ABC transporters in bacteria and cancer cells. Ponte-Sucre A. ABC transporters in microorganisms, Caister Academic Press, UK2009. pp. 35-50.

[12] Callaghan R, Crowley E, Potter S, Kerr ID. P-glycoprotein: So Many Ways to Turn It On. J Clin Pharmacol 2008; 48: 365-78.

[13] Penson RT, Oliva E, Skates SJ, *et al*. Expression of multidrug resistance-1 protein inversely correlates with paclitaxel response and survival in ovarian cancer patients: a study in serial samples. Gynecol Oncol 2004; 93: 98-106.

[14] Chan HS, Haddad G, Thorner PS, *et al*. P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma. N Engl J Med 1991; 325: 1608-14.

[15] Allikmets R, Schriml LM, Hutchinson A, Romano-Spica V, Dean M. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. Cancer Res 1998; 58: 5337-9.

[16] Doyle LA, Yang W, Abruzzo LV, *et al*. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci USA 1998; 95: 15665-70.

[17] Miyake K, Mickley L, Litman T, *et al*. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. Cancer Res 1999; 59: 8-13.

[18] Bauer BE, Wolfger H, Kuchler K. Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. Biochim Biophys Acta-Biomembr 1999; 1461: 217-36.

[19] Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood 2002; 99: 507-12.

[20] Noguchi K, Katayama K, Mitsuhashi J, Sugimoto Y. Functions of the breast cancer resistance protein (BCRP/ABCG2) in chemotherapy. Adv Drug Deliv Rev 2009; 61: 26-33.

[21] Robey RW, Polgar O, Deeken J, To KW, Bates SE. ABCG2: determining its relevance in clinical drug resistance. Cancer Metastasis Rev 2007; 26: 39-57.

[22] Cole SP, Bhardwaj G, Gerlach JH, *et al*. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 1992; 258: 1650-4.

[23] Eckford PD, Sharom FJ. ABC efflux pump-based resistance to chemotherapy drugs. Chem Rev 2009; 109: 2989-3011.

[24] Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. J Natl Cancer Inst 2000; 92: 1295-302.

[25] Ishikawa T, Kuo MT, Furuta K, Suzuki M. The human multidrug resistance-associated protein (MRP) gene family: from biological function to drug molecular design. Clin Chem Lab Med 2000; 38: 893-7.

[26] Evers R, Zaman GJR, Van Deemter L, *et al*. Basolateral localization and export activity of the human multidrug resistance - associated protein in polarized pig kidney cells. J Clin Invest 1996; 97: 1211-8.

[27] Hipfner DR, Deeley RG, Cole SP. Structural, mechanistic and clinical aspects of MRP1. Biochim Biophys Acta 1999; 1461: 359-76.

[28] Stewart AJ, Canitrot Y, Baracchini E, Dean NM, Deeley RG, Cole SP. Reduction of expression of the multidrug resistance protein (MRP) in human tumor cells by antisense phosphorothioate oligonucleotides. Biochem Pharmacol 1996; 51: 461-9.

[29] Bordow SB, Haber M, Madafiglio J, Cheung B, Marshall GM, Norris MD. Expression of the multidrug resistance-associated protein (MRP) gene correlates with amplification and overexpression of the N-myc oncogene in childhood neuroblastoma. Cancer Res 1994; 54: 5036-40.

[30] Nooter K, Brutel de la Riviere G, Look MP, *et al*. The prognostic significance of expression of the multidrug resistance-associated protein (MRP) in primary breast cancer. Br J Cancer 1997; 76: 486-93.

[31] Arts HJ, Katsaros D, de Vries EG, *et al*. Drug resistance-associated markers P-glycoprotein, multidrug resistance-associated protein 1, multidrug resistance-associated protein 2, and lung resistance protein as prognostic factors in ovarian carcinoma. Clin Cancer Res 1999; 5: 2798-805.

[32] Goldberg RM, Sargent DJ, Morton RF, *et al*. A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 2004; 22: 23-30.

[33] Ma WW, Adjei AA. Novel agents on the horizon for cancer therapy. CA Cancer J Clin 2009; 59: 111-37.

[34] Shukla S, Wu CP, Ambudkar SV. Development of inhibitors of ATP-binding cassette drug transporters: present status and challenges. Expert Opin Drug Metab Toxicol 2008; 4: 205-23.

[35] Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y. Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res 1981; 41: 1967-72.

[36] Slater LM, Sweet P, Stupecky M, Gupta S. Cyclosporin A reverses vincristine and daunorubicin resistance in acute lymphatic leukemia *in vitro*. J Clin Invest 1986; 77: 1405-8.

[37] Cornwell MM, Pastan I, Gottesman MM. Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. J Biol Chem 1987; 262: 2166-70.

[38] Foxwell BM, Mackie A, Ling V, Ryffel B. Identification of the multidrug resistance-related P-glycoprotein as a cyclosporine binding protein. Mol Pharmacol 1989; 36: 543-6.

[39] Goldberg H, Ling V, Wong PY, Skorecki K. Reduced cyclosporin accumulation in multidrug-resistant cells. Biochem Biophys Res Commun 1988; 152: 552-8.

[40] Twentyman PR, Bleehen NM. Resistance modification by PSC-833, a novel non-immunosuppressive cyclosporin [corrected]. Eur J Cancer 1991; 27: 1639-42.

[41] Benson AB, 3rd, Trump DL, Koeller JM, *et al*. Phase I study of vinblastine and verapamil given by concurrent iv infusion. Cancer Treat Rep 1985; 69: 795-9.

[42] Verweij J, Herweijer H, Oosterom R, *et al*. A phase II study of epidoxorubicin in colorectal cancer and the use of cyclosporin-A in an attempt to reverse multidrug resistance. Br J Cancer 1991; 64: 361-4.

[43] Bradshaw DM, Arceci RJ. Clinical relevance of transmembrane drug efflux as a mechanism of multidrug resistance. J Clin Oncol 1998; 16: 3674-90.

[44] Friedenberg WR, Rue M, Blood EA, *et al*. Phase III study of PSC-833 (valspodar) in combination with vincristine, doxorubicin, and dexamethasone (valspodar/VAD) versus VAD alone in patients with recurring or refractory multiple myeloma (E1A95): a trial of the Eastern Cooperative Oncology Group. Cancer 2006; 106: 830-8.

[45] Roe M, Folkes A, Ashworth P, *et al*. Reversal of P-glycoprotein mediated multidrug resistance by novel anthranilamide derivatives. Bioorg Med Chem Lett 1999; 9: 595-600.

[46] Hyafil F, Vergely C, Du Vignaud P, Grand-Perret T. *In vitro* and *in vivo* reversal of multidrug resistance by GF120918, an acridonecarboxamide derivative. Cancer Res 1993; 53: 4595-602.

[47] Dantzig AH, Shepard RL, Cao J, *et al*. Reversal of P-glycoprotein-mediated multidrug resistance by a potent cyclopropyldibenzo-suberane modulator, LY335979. Cancer Res 1996; 56: 4171-9.

[48] Naito M, Matsuba Y, Sato S, Hirata H, Tsuruo T. MS-209, a quinoline-type reversal agent, potentiates antitumor efficacy of docetaxel in multidrug-resistant solid tumor xenograft models. Clin Cancer Res 2002; 8: 582-8.

[49] Martin C, Berridge G, Mistry P, Higgins C, Charlton P, Callaghan R. The molecular interaction of the high affinity reversal agent XR9576 with P-glycoprotein. Br J Pharmacol 1999; 128: 403-11.

[50] Mistry P, Stewart AJ, Dangerfield W, *et al*. *In vitro* and *in vivo* reversal of P-glycoprotein-mediated multidrug resistance by a novel potent modulator, XR9576. Cancer Res 2001; 61: 749-58.

[51] Fox E, Bates SE. Tariquidar (XR9576): a P-glycoprotein drug efflux pump inhibitor. Expert Rev Anticancer Ther 2007; 7: 447-59.

[52] Abraham J, Edgerly M, Wilson R, *et al*. A phase I study of the P-glycoprotein antagonist tariquidar in combination with vinorelbine. Clin Cancer Res 2009; 15: 3574-82.

[53] Gekeler V, Ise W, Sanders KH, Ulrich WR, Beck J. The leukotriene LTD4 receptor antagonist Mk571 specifically modulates MRP associated multidrug resistance. Biochem Biophys Res Commun 1995; 208: 345-52.

[54] Rabindran SK, Ross DD, Doyle LA, Yang W, Greenberger LM. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. Cancer Res 2000; 60: 47-50.

[55] Germann UA, Shlyakhter D, Mason VS, *et al*. Cellular and biochemical characterization of VX-710 as a chemosensitizer: reversal of P-glycoprotein-mediated multidrug resistance *in vitro*. Anticancer Drugs 1997; 8: 125-40.

[56] Minderman H, O'Loughlin KL, Pendyala L, Baer MR. VX-710 (biricodar) increases drug retention and enhances chemosensitivity in resistant cells overexpressing P-glycoprotein, multidrug resistance protein, and breast cancer resistance protein. Clin Cancer Res 2004; 10: 1826-34.

[57] Seiden MV, Swenerton KD, Matulonis U, *et al*. A phase II study of the MDR inhibitor biricodar (INCEL, VX-710) and paclitaxel in women with advanced ovarian cancer refractory to paclitaxel therapy. Gynecol Oncol 2002; 86: 302-10.

[58] Toppmeyer D, Seidman AD, Pollak M, *et al*. Safety and efficacy of the multidrug resistance inhibitor Incel (biricodar; VX-710) in combination with paclitaxel for advanced breast cancer refractory to paclitaxel. Clin Cancer Res 2002; 8: 670-8.

[59] Gandhi L, Harding MW, Neubauer M, *et al*. A phase II study of the safety and efficacy of the multidrug resistance inhibitor VX-710 combined with doxorubicin and vincristine in patients with recurrent small cell lung cancer. Cancer 2007; 109: 924-32.

[60] Robey RW, Shukla S, Finley EM, *et al*. Inhibition of P-glycoprotein (ABCB1)- and multidrug resistance-associated protein 1 (ABCC1)-mediated transport by the orally administered inhibitor, CBT-1((R)). Biochem Pharmacol 2008; 75: 1302-12.

[61] Robey RW, To KK, Polgar O, *et al*. ABCG2: a perspective. Adv Drug Deliv Rev 2009; 61: 3-13.

[62] Mechetner EB, Roninson IB. Efficient inhibition of P-glycoprotein-mediated multidrug resistance with a monoclonal antibody. Proc Natl Acad Sci USA 1992; 89: 5824-8.

[63] Widmer N, Rumpold H, Untergasser G, Fayet A, Buclin T, Decosterd LA. Resistance reversal by RNAi silencing of MDR1 in CML cells associated with increase in imatinib intracellular levels. Leukemia 2007; 21: 1561-2.

[64] Jin S, Gorfajn B, Faircloth G, Scotto KW. Ecteinascidin 743, a transcription-targeted chemotherapeutic that inhibits MDR1 activation. Proc Natl Acad Sci USA 2000; 97: 6775-9.

[65] Oostendorp RL, Beijnen JH, Schellens JHM. The biological and clinical role of drug transporters at the intestinal barrier. Cancer Treat Rev 2009; 35: 137-47.

[66] Sparreboom A, van Asperen J, Mayer U, *et al*. Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. Proc Natl Acad Sci USA 1997; 94: 2031-5.

[67] Greiner B, Eichelbaum M, Fritz P, *et al*. The role of intestinal P-glycoprotein in the interaction of digoxin and rifampin. J Clin Invest 1999; 104: 147-53.

[68] Shukla S, Zaher H, Hartz A, Bauer B, Ware J, Ambudkar S. Curcumin inhibits the activity of ABCG2/BCRP1, a multidrug resistance-linked ABC drug transporter in mice. Pharm Res 2009; 26: 480-7.

[69] Kwak J-O, Lee SH, Lee GS, *et al*. Selective inhibition of MDR1 (ABCB1) by HM30181 increases oral bioavailability and therapeutic efficacy of paclitaxel. Eur J Pharmacol 2010; 627: 92-8.

[70] Yasuda K, Lan L-B, Sanglard D, Furuya K, Schuetz JD, Schuetz EG. Interaction of Cytochrome P450 3A Inhibitors with P-Glycoprotein. J Pharmacol Exp Ther 2002; 303: 323-32.

[71] Loscher W, Potschka H. Drug resistance in brain diseases and the role of drug efflux transporters. Nat Rev Neurosci 2005; 6: 591-602.

[72] Schinkel AH, Wagenaar E, Mol CA, van Deemter L. P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. J Clin Invest 1996; 97: 2517-24.

[73] Schinkel AH, Smit JJM, Van Tellingen O, *et al*. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell 1994; 77: 491-502.

[74] Mayer U, Wagenaar E, Dorobek B, Beijnen JH, Borst P, Schinkel AH. Full blockade of intestinal P-glycoprotein and extensive inhibition of blood-brain barrier P-glycoprotein by oral treatment of mice with PSC833. J Clin Invest 1997; 100: 2430-6.

[75] Miyama T, Takanaga H, Matsuo H, *et al*. P-glycoprotein-mediated transport of itraconazole across the blood- brain barrier. Antimicrob Agents Chemother 1998; 42: 1738-44.

[76] Kawahara M, Sakata A, Miyashita T, Tamai I, Tsuji A. Physiologically based pharmacokinetics of digoxin in mdr1a knockout mice. J Pharm Sci 1999; 88: 1281-7.

[77] de Vries NA, Zhao J, Kroon E, Buckle T, Beijnen JH, van Tellingen O. P-Glycoprotein and Breast Cancer Resistance Protein: Two Dominant Transporters Working Together in Limiting the Brain Penetration of Topotecan. Clin Cancer Res 2007; 13: 6440-9.

[78] Lagas JS, van Waterschoot RAB, van Tilburg VACJ, *et al*. Brain accumulation of dasatinib is restricted by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) and can be enhanced by elacridar treatment. Clin Cancer Res 2009; 15: 2344-51.

[79] Breedveld P, Pluim D, Cipriani G, *et al*. The effect of Bcrp1 (Abcg2) on the *in vivo* pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients. Cancer Res 2005; 65: 2577-82.

[80] Bihorel S, Camenisch G, Lemaire M, Scherrmann J-M. Modulation of the Brain Distribution of Imatinib and its Metabolites in Mice by Valspodar, Zosuquidar and Elacridar. Pharm Res 2007; 24: 1720-8.

[81] Morschhauser F, Zinzani PL, Burgess M, Sloots L, Bouafia F, Dumontet C. Phase I/II trial of a P-glycoprotein inhibitor, Zosuquidar. 3HCl trihydrochloride (LY335979), given orally in combination with the CHOP regimen in patients with non-Hodgkin's lymphoma. Leuk Lymphoma 2009; 48: 708-15.

[82] Kuppens IELM, Witteveen EO, Jewell RC, *et al*. A phase I, randomized, open-label, parallel-cohort, dose-finding study of elacridar (GF120918) and oral topotecan in cancer patients. Clin Cancer Res 2007; 13: 3276-85.

[83] Saeki T, Nomizu T, Toi M, *et al*. Dofequidar fumarate (MS-209) in combination with cyclophosphamide, doxorubicin, and fluorouracil for patients with advanced or recurrent breast cancer. J Clin Oncol 2007; 25: 411-7.

[84] Oostendorp RL, Huitema A, Rosing H, *et al*. Coadministration of ritonavir strongly enhances the apparent oral bioavailability of docetaxel in patients with solid tumors. Clin Cancer Res 2009; 15: 4228-33.

[85] Lhomme C, Joly F, Walker JL, *et al*. Phase III study of valspodar (PSC 833) combined with paclitaxel and carboplatin compared with paclitaxel and carboplatin alone in patients with stage IV or suboptimally debulked stage III epithelial ovarian cancer or primary peritoneal cancer. J Clin Oncol 2008; 26: 2674-82.

[86] Fung KL, Gottesman MM. A synonymous polymorphism in a common MDR1 (ABCB1) haplotype shapes protein function. Biochimica et Biophysica Acta (BBA) - Proteins Proteom 2009; 1794: 860-71.

[87] Sissung T, Baum C, Kirkland C, Gao R, Gardner E, Figg W. Pharmacogenetics of membrane transporters: an update on current approaches. Mol Biotechnol 2010; 44: 152-67.

[88] Kimchi-Sarfaty C, Oh JM, Kim I-W, *et al*. A "Silent" polymorphism in the MDR1 gene changes substrate specificity. Science 2007; 315: 525-8.

[89] Sparreboom A, Loos WJ, Burger H, *et al*. Effect of ABCG2 genotype on the oral bioavailability of topotecan. Cancer Biol Ther 2005; 4: 650-8.

[90] Gardner ER, Ahlers CM, Shukla S, *et al*. Association of the ABCG2 C421A polymorphism with prostate cancer risk and survival. BJU Int 2008; 102: 1694-9.

[91] Keskitalo JE, Zolk O, Fromm MF, Kurkinen KJ, Neuvonen PJ, Niemi M. ABCG2 polymorphism markedly affects the pharmacokinetics of atorvastatin and rosuvastatin. Clin Pharmacol Ther 2009; 86: 197-203.

[92] Crettol S, Deglon J-J, Besson J, *et al*. ABCB1 and cytochrome P450 genotypes and phenotypes: influence on methadone plasma levels and response to treatment[ast]. Clin Pharmacol Ther 2006; 80: 668-81.

[93] van Asperen J, van Tellingen O, Sparreboom A, *et al*. Enhanced oral bioavailability of paclitaxel in mice treated with the P-glycoprotein blocker SDZ PSC 833. Br J Cancer 1997; 76: 1181-3.

[94] Spahn-Langguth H, Baktir G, Radschuweit A, *et al*. P-glycoprotein transporters and the gastrointestinal tract: Evaluation of the potential *in vivo* relevance of *in vitro* data employing talinolol as model compound. Int J Clin Pharmacol Ther 1998; 36: 16-24.

[95] Sean XP, David MR, Martin C, Earl D, Robert D, Jane F. Altered oral bioavailability and pharmacokinetics of P-glycoprotein substrates by coadministration of biochanin A. J Pharm Sci 2006; 95: 1984-93.

[96] Bardelmeijer HA, Beijnen JH, Brouwer KR, *et al*. Increased oral bioavailability of paclitaxel by GF120918 in mice through selective modulation of P-glycoprotein. Clin Cancer Res 2000; 6: 4416-21.

[97] Van Asperen J, Van Tellingen O, Van Der Valk MA, Rozenhart M, Beijnen JH. Enhanced oral absorption and decreased elimination of paclitaxel in mice cotreated with cyclosporin A. Clin Cancer Res 1998; 4: 2293-7.

[98] Bardelmeijer HA, Ouwehand M, Buckle T, *et al*. Low systemic exposure of oral docetaxel in mice resulting from extensive first-pass metabolism is boosted by ritonavir. Cancer Res 2002; 62: 6158-64.

[99] Meerum Terwogt JM, Malingre MM, *et al*. Coadministration of oral cyclosporin A enables oral therapy with paclitaxel. Clin Cancer Res 1999; 5: 3379-84.

[100] Doering W. Quinidine-digoxin interaction. Pharmacokinetics, underlying mechanism and clinical implications. N Engl J Med 1979; 301: 400-4.

[101] Malingre MM, Richel DJ, Beijnen JH, *et al*. Coadministration of cyclosporine strongly enhances the oral bioavailability of docetaxel. J Clin Oncol 2001; 19: 1160-6.

[102] Westphal K, Weinbrenner A, Giessmann T, *et al*. Oral bioavailability of digoxin is enhanced by talinolol: Evidence for involvement of intestinal P-glycoprotein. Clin Pharmacol Ther 2000; 68: 6-12.

[103] Malingre MM, Beijnen JH, Rosing H, *et al*. Co-administration of GF120918 significantly increases the systemic exposure to oral paclitaxel in cancer patients. Br J Cancer 2001; 84: 42-7.

[104] Kruijtzer CM, Beijnen JH, Rosing H, *et al*. Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. J Clin Oncol 2002; 20: 2943-50.

[105] Allen JD, Van Dort SC, Buitelaar M, van Tellingen O, Schinkel AH. Mouse breast cancer resistance protein (Bcrp1/Abcg2) mediates etoposide resistance and transport, but etoposide oral availability is limited primarily by P-glycoprotein. Cancer Res 2003; 63: 1339-44.

[106] Kruijtzer CMF, Beijnen JH, Rosing H, *et al*. Increased Oral Bioavailability of Topotecan in Combination With the Breast Cancer Resistance Protein and P-Glycoprotein Inhibitor GF120918. J Clin Oncol 2002; 20: 2943-50.

[107] Breedveld P, Zelcer N, Pluim D, *et al*. Mechanism of the pharmacokinetic interaction between methotrexate and benzimidazoles: Potential role for breast cancer resistance protein in clinical drug-drug interactions. Cancer Res 2004; 64: 5804-11.

[108] Stewart CF, Leggas M, Schuetz JD, *et al*. Gefitinib enhances the antitumor activity and oral bioavailability of irinotecan in mice. Cancer Res 2004; 64: 7491-9.

[109] Joerger M, Huitema ADR, Van Den Bongard HJGD, *et al*. Determinants of the elimination of methotrexate and 7-hydroxy-methotrexate following high-dose infusional therapy to cancer patients. Br J Clin Pharmacol 2006; 62: 71-80.

[110] Marchetti S, de Vries NA, Buckle T, *et al*. Effect of the ATP-binding cassette drug transporters ABCB1, ABCG2, and ABCC2 on erlotinib hydrochloride (Tarceva) disposition in *in vitro* and *in vivo* pharmacokinetic studies employing Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ (triple-knockout) and wild-type mice. Mol Cancer Ther 2008; 7: 2280-7.

[111] Leggas M, Panetta JC, Zhuang Y, *et al*. Gefitinib modulates the function of multiple ATP-binding cassette transporters *in vivo*. Cancer Res 2006; 66: 4802-7.

[112] Sparreboom A, van Asperen J, Mayer U, *et al*. Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. Proc Natl Acad Sci USA 1997; 94: 2031-5.

[113] Mitsunaga Y, Takanaga H, Matsuo H, *et al*. Effect of bioflavonoids on vincristine transport across blood-brain barrier. Eur J Pharmacol 2000; 395: 193-201.

[114] Cisternino S, Rousselle C, Dagenais C, Scherrmann JM. Screening of multidrug-resistance sensitive drugs by in situ brain perfusion in P-glycoprotein-deficient mice. Pharm Res 2001; 18: 183-90.

[115] Drion N, Lemaire M, Lefauconnier J-M. Role of P-glycoprotein in the blood-brain transport of colchicine and vinblastine. J Neurochem 1996; 67: 1688-93.

[116] Drion N, Risede P, Cholet N, Chanez C, Scherrmann JM. Role of P-170 glycoprotein in colchicine brain uptake. J Neurosci Res 1997; 49: 80-8.

[117] Desrayaud S, Guntz P, Scherrmann JM, Lemaire M. Effect of the P-glycoprotein inhibitor, SDZ PSC 833, on the blood and brain pharmacokinetics of colchicine. Life Sci 1997; 61: 153-63.

[118] Kemper EM, van Zandbergen AE, Cleypool C, *et al*. Increased penetration of paclitaxel into the brain by inhibition of P-glycoprotein. Clin Cancer Res 2003; 9: 2849-55.

[119] Kemper EM, Cleypool C, Boogerd W, Beijnen J, Tellingen O. The influence of the P-glycoprotein inhibitor zosuquidar trihydrochloride (LY335979) on the brain penetration of paclitaxel in mice. Cancer Chemother Pharmacol 2004; 53: 173-8.

[120] Fellner S, Bauer B, Miller DS, *et al*. Transport of paclitaxel (Taxol) across the blood-brain barrier *in vitro* and *in vivo*. J Clin Invest 2002; 110: 1309-18.

[121] Chen Y, Agarwal S, Shaik NM, Chen C, Yang Z, Elmquist WF. P-glycoprotein and breast cancer resistance protein influence brain distribution of dasatinib. J Pharmacol Exp Ther 2009; 330: 956-63.

[122] Bihorel S, Camenisch G, Lemaire M, Scherrmann JM. Influence of breast cancer resistance protein (Abcg2) and p-glycoprotein (Abcb1a) on the transport of imatinib mesylate (Gleevec) across the mouse blood-brain barrier. J Neurochem 2007; 102: 1749-57.

[123] Lee YJ, Kusuhara H, Jonker JW, Schinkel AH, Sugiyama Y. Investigation of efflux transport of dehydroepiandrosterone sulfate and mitoxantrone at the mouse blood-brain barrier: a minor role of breast cancer resistance protein. J Pharmacol Exp Ther 2005; 312: 44-52.

[124] Cisternino S, Mercier C, Bourasset F, Roux F, Scherrmann JM. Expression, up-regulation, and transport activity of the multidrug-resistance protein Abcg2 at the mouse blood-brain barrier. Cancer Res 2004; 64: 3296-301.

Received: December 31, 2009 Revised: March 18, 2010 Accepted: March 18, 2010

Genetic Polymorphisms of ATP-Binding Cassette Transporters ABCB1 and ABCC2 and their Impact on Drug Disposition

Vincent Haufroid*[,1,2]

[1]*Louvain Centre for Toxicology and Applied Pharmacology (LTAP), Université catholique de Louvain, Avenue E. Mounier 53.02 and* [2]*Laboratory of Analytical Chemistry, Saint-Luc Hospital, Avenue Hippocrate 10, 1200 Brussels, Belgium*

Abstract: The ATP-binding cassette (ABC) transporter superfamily comprises membrane proteins that translocate a variety of substrates across extra- and intra-cellular membranes, and act as efflux proteins. ABC transporters are characterised by the presence of genetic polymorphisms mainly represented by single nucleotide polymorphisms (SNPs), some of which having an impact on their activity. Besides physiological substances, drugs are also substrates of some ABC transporters, mainly ABCB1, ABCC1, ABCC2, ABCC3 and ABCG2. Identifying the impact of these polymorphisms on the pharmacokinetics (PK) of these drugs may have important clinical implications, certainly for those characterised by a narrow therapeutic index and significant inter- and intra-patient PK variability. This review focuses specifically on ABCB1 and ABCC2 and critically analyses important publications dealing with the influence of *ABCB1* and/or *ABCC2* polymorphisms on drug disposition in humans. For different reasons discussed in this paper, the effect of *ABCB1* and/or *ABCC2* polymorphisms on drug concentrations in blood is not always easy to interpret and to correlate with pharmacological effects. In contrast, intracellular or target tissue drug concentrations appear more directly influenced by these polymorphisms, as illustrated with intralymphocyte concentrations for immunosupressants and antiretrovirals or with cerebrospinal fluid (CSF) concentrations for antiepileptics and antidepressants. Further research on intracellular and/or target tissue drug concentrations are still needed to better characterise the PK-PG (pharmacogenetics) relationship involving ABC transporters.

Keywords: Pharmacogenomics, ABCB1, ABCC2, intracellular concentrations, genotype, polymorphism, mdr1, mrp2.

INTRODUCTION

The ATP-binding cassette (ABC) transporter superfamily comprises membrane proteins that translocate a variety of substrates across extra- and intra-cellular membranes, and act as efflux proteins. In humans, 48 different ABC transporters have been identified and categorised into seven distinct subfamilies on the basis of their sequence homology and domain organisation. Their physiological and pharmacological functions have been extensively reviewed [1]. As any other protein, ABC transporters are characterised by genetic polymorphisms, mainly single nucleotide polymorphisms (SNPs), consisting in a variation of a single nucleotide (substitution, deletion or insertion) within the DNA sequence, some of which having an impact on the activity of the protein. Besides physiological substances, drugs are also substrates of some ABC transporters, mainly ABCB1, ABCC1, ABCC2, ABCC3 and ABCG2. Identifying the impact of these polymorphisms on the pharmacokinetics (PK) of these drugs may have important clinical implications. Indeed, the clinical use of several drugs is complicated by a narrow therapeutic index, a significant inter- and intra-patient PK variability, and the potential for serious drug interactions. For these drugs, therapeutic drug monitoring (TDM) and dosage individualisation are therefore highly recommended to optimise efficacy and reduce toxicity. In the daily practice, the conventional TDM is most frequently based on the measurement of trough whole blood or plasma concentrations (C0), just before the next dose, followed by drug dosage adjustment according to the concentration measured. Several polymorphisms in genes coding for biotransformation enzymes and/or transport proteins may significantly contribute to this variability, and genotyping for some key polymorphisms may assist the clinician in the interpretation of TDM and/or to anticipate dosage adjustment [2].

This review focuses specifically on ABCB1 and ABCC2 and critically analyses important publications having investigated the impact of *ABCB1* and *ABCC2* polymorphisms on drug disposition in humans. Relevant studies were selected by searching in PubMed (up to August 2009) with the following selection criteria ((abcb1 OR mdr1) AND (genotype OR polymorphism)) and ((abcc2 OR mrp2) AND (genotype OR polymorphism)) for ABCB1 and ABCC2, respectively. Only papers dealing with the influence of *ABCB1* and/or *ABCC2* polymorphisms on drug PK parameters were taken into account (PK-PG analyses), excluding those investigating only the association between genetic polymorphisms and clinical endpoints such as drug efficacy and/or drug toxicity. In addition, given the wide organ distribution of both ABC transporters, it must be already kept in mind that a given SNP in *ABCB1* or *ABCC2* is not expected to have an effect on a single physiological process but, on the contrary, would be more susceptible to influence a number of processes such as drug absorption (in gut), elimination (in liver and kidney) and distribution (in blood-brain, blood-heart-barriers, etc.), each process potentially influencing drug distribution in an independent manner (Fig. **1**). Therefore, the resulting effect on blood PK parameters may be difficult to predict due to the complexity of those various biological barriers.

*Address correspondence to this author at the Laboratory of Analytical Chemistry, Saint-Luc Hospital, Avenue Hippocrate 10, 1200 Brussels, Belgium; Tel: +32.2.764.67.25; E-mail: Vincent.Haufroid@uclouvain.be

1389-4501/11 $58.00+.00 © 2011 Bentham Science Publishers Ltd.

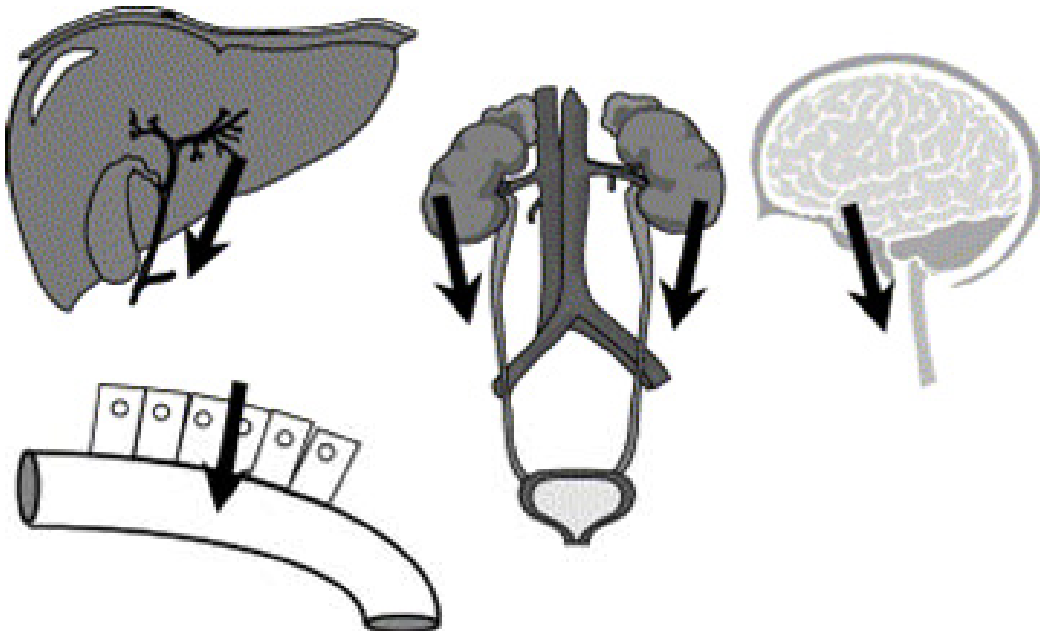


Fig. (1). Expression and function of ABCB1 in selected tissues. The arrows indicate the direction of ABCB1-mediated transport, which leads to reduced intracellular concentrations of ABCB1 substrates (with permission from Fromm [153]).

ABCB1

The ABCB1 transporter, also known as P-glycoprotein (P-gp), was first described in tumour cells that were becoming resistant to various anticancer drugs as a result of its over-expression. For this reason, ABCB1 was first named *multidrug resistance protein 1* (MDR1) before the proposal of an official nomenclature for the ABC transporters by the Human Genome Organization (HUGO) in October 1999 (http://www.genenames.org/genefamily/abc.html). Several SNPs have been reported for the *ABCB1 (MDR1)* gene, some of which affecting P-gp expression and/or function (see ABCB1 on http://www.pharmgkb.org/search/) (Table **1**). Three among those SNPs, namely1236C>T (rs1128503), 2677G>T/A (rs2032582) and 3435C>T (rs1045642), located in exons 12, 21 and 26, respectively, are in strong linkage disequilibrium and have been studied extensively. Interestingly, these SNPs are very frequent in most ethnic groups [3, 4] and only the 2677G>T/A polymorphism results in an amino acid substitution (Ala893Ser/Thr), whereas 1236C>T and 3435C>T are synonymous SNPs. The SNP in exon 26 (i.e. 3435C>T), resulting in a silent mutation, has probably been the most investigated so far. The initial study reporting an effect of this polymorphism was already published 10 years ago [5]. In this study, homozygous carriers of the *ABCB1* 3435T allele had on average more than two-fold lower intestinal P-gp expression levels, measured by western blot analysis, compared to homozygous carriers of the 3435C allele. In accordance with such an effect at the protein level, the 3435C>T polymorphism has been associated with reduced mRNA expression and stability *ex vivo* [6] but this finding could not be confirmed *in vitro* [7]. More recently, this polymorphism has been associated with changes in substrate specificity [7]. In this latter publication, the authors suggested that the presence of the rare codon, marked by the synonymous polymorphism, could affect the timing of cotranslational folding and the insertion of P-gp into the membrane, thereby altering the structure of substrate and inhibitor interaction sites. Altogether, based on existing data, the *in vivo* impact of the 3435C>T polymorphism could therefore involve effects, at both transcriptional and translational levels, affecting not only P-gp expression but also substrate specificity, rending the effect of this SNP on drug disposition difficult to predict. Therefore, it is also

Table 1. Important Polymorphisms (SNPs) in *ABCB1* Gene Selected in PharmGKB Database (http://www.pharmgkb.org/search/)

Position	Nucleotide Change	Effect[a]	NCBI dbSNP	Allelic Frequency[b]
Exon 2	61A>G	Asn21Asp	rs9282564	Ca: 0.08 As: 0.05 Af: 0.03
Exon 11	1199G>A	Ser400Asn	rs2229109	Ca: 0.03 As: 0.00 Af: 0.01
Exon 12	1236C>T	synonymous	rs1128503	Ca: 0.46 As: 0.67 Af: 0.21
Exon 21	2677G>T 2677G>A	Ala893Ser Ala893Thr	rs2032582	Ca: 0.38 As: 0.45 Af: 0.10 Ca: 0.02 As: 0.20 Af: 0.01
Exon 26	3435C>T	synonymous	rs1045642	Ca: 0.56 As: 0.45 Af: 0.20

NCBI: National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/projects/SNP/)
[a]number represents amino-acid codon
[b]based on information found in http://www.pharmgkb.org/search/
Ca: Caucasian, As: Asian, Af: African

Table 2. Impact of *ABCB1* Polymorphisms on PK Parameters of Selected Drugs

Drug	Polymorphism Investigated (exon)	PK Parameter	Effect	Refs.
Probe drug				
Digoxin	3435	Cmax (1)	higher Cmax in 3435TT	[5]
	3435	AUC	n.s. (meta analysis*)	[11]
	3435	Cmax	higher Cmax in 3435TT (meta analysis*)	[11]
	2677, 3435	AUC, Cmax (1)	higher AUC and Cmax in 3435TT	[16]
	2677, 3435	Vd (1)	lower Vd in 3435TT	[17]
	1236, 2677, 3435	C0 (2)	higher C0 in 3435TT and in T-T-T homozygous	[18]
	2677, 3435	C0 (2)	n.s.	[19]
Immunosupp-ressant				
	3435	AUC0-4	n.s. (meta analysis*)	[20]
Cyclosporin	3435	AUC0-12	higher AUC0-12 in 3435T carriers (meta analysis*)	[20]
	3435	Cmax	n.s. (meta analysis*)	[20]
	3435	C0	higher C0 in 3435T carriers (meta analysis*)	[20]
	61, 1199, 1236, 2677, 3435	PBMC (2)	lower PBMC concentration in 1199A carriers higher PBMC concentration in 3435T carriers	[31]
Tacrolimus	1236, 2677, 3435	C0, d.req. (2)	n.s.	[28]
	1236, 2677, 3435	C0, d.req. (2)	n.s.	[29]
	2677, 3435	C0, d.req. (2)	n.s.	[35]
	3435	C0 (2)	n.s.	[36]
	1236, 2677, 3435	C0, d.req. (1)	n.s.	[37]
	1236, 2677, 3435	AUC, Cmax (1)	n.s.	[38]
	1236, 2677, 3435	AUC (2)	n.s.	[39]
	3435	C0 (2)	n.s.	[40]
	1236, 2677, 3435	AUC (2)	n.s.	[41]
	2677, 3435	d.req. (2)	n.s.	[42]
	2677, 3435	C0, d.req. (2)	n.s.	[43]
	2677, 3435	C0 (2)	n.s.	[44]
	2677, 3435	C0 (2)	higher C0 in 3435T carriers	[45]
	2677, 3435	C0, d.req. (2)	higher C0 in 3435T carriers	[46]
	3435	C0 (2)	higher C0 in 3435T carriers	[47]
	3435	C0 (2)	higher C0 in 3435T carriers	[48]
	1236, 2677, 3435	C0, d.req. (2)	lower C0 and higher dose req. in 2677GG	[49]
	2677, 3435	AUC (2)	higher AUC in 3435T carriers (only trend)	[50]
	3435	C0, d.req. (2)	lower dose req. in 3435TT	[51]
	3435	C0 (2)	lower C0 in 3435CC	[53]
	1236, 2677, 3435	AUC, C0 (1)	n.s.**	[2]
	3435	C0, d.req. (2)	n.s.**	[54]
	1236, 2677, 3435	C0, d.req. (2)	n.s.**	[55]
	1236, 2677, 3435	C0 (2)	lower C0 in C-G-C homozygous**	[56]
	1199, 1236, 2677, 3435	hepatic conc. (2)	higher hepatic concentration in 1199A or 3435T carriers	[58]
Sirolimus	1236, 2677, 3435	C0 (2)	n.s.	[59]
	1236, 2677, 3435	C0, d.req. (2)	n.s.	[60]
	2677, 3435	AUC, CL (2)	n.s.	[61]
	3435	C0 (2)	n.s.	[62]
Anti-cancer				
Docetaxel	3435	AUC, CL (2)	n.s.	[63]
	3435	AUC, CL (2)	n.s.	[64]
	1236, 2677, 3435	CL (2)	lower CL in 1236TT	[65]
Paclitaxel	3435	AUC (2)	higher AUC in 3435T carriers for main metabolite	[66]
	3435	CL (2)	n.s.	[67]
	2677, 3435	CL (2)	n.s.	[68]
Vincristine	2677, 3435	AUC, CL (2)	n.s.	[69]
Irinotecan	1236, 2677, 3435	CL (2)	lower CL in T-T-T haplotype	[70]
	1233, 2677, 3435	AUC, CL (2)	higher AUC in C-G-C haplotype for metabolite SN-38	[71]

(Table 2) Contd…..

Drug	Polymorphism Investigated (exon)	PK parameter	Effect	Refs.
Imatinib	3435	CL (2)	n.s.	[72]
	1236, 2677, 3435	CL (2)	lower reduction in CL between day 1 and SS in T-T-T homozygous	[73]
	1236, 2677, 3435	C0 (2)	higher C0 in 1236TT	[74]
Doxorubicin	1236, 2677, 3435	AUC, CL (2)	lower AUC and higher CL in C-G-C homozygous	[75]
Anti-infective				
Lopinavir	3435	C0 (2)	n.s.	[76]
	2677, 3435	C0 (2)	n.s.	[77]
	1236, 2677, 3435	C0 (2)	n.s.	[78]
Saquinavir	2677, 3435	AUC, CL Cmax (1)	n.s.	[79]
	2677, 3435	AUC, CL, Cmax (1)	n.s.	[80]
	1236, 2677, 3435	AUC, CL (1)	n.s.	[81]
Indinavir	2677, 3435	C0 (2)	n.s.	[82]
	3435	k_a (2)	lower k_a in 3435CC *vs*. 3435CT	[83]
	3435	Vd, CL (2)	higher Vd and CL in 3435CC	[84]
Atazanavir	3435	C0 (2)	higher C0 in 3435CC	[85]
	3435	C0 (2)	higher C0 in 3435CC	[86]
	2677, 3435	C0 (2)	n.s.	[77]
Nelfinavir	3435	C0 (2)	n.s.	[87]
	3435	C0 (2)	n.s. (higher C0 in 3435CT only)	[88]
	2677, 3435	PBMC (2)	higher PBMC concentration in 3435TT	[89]
Nevirapine	3435	C0 (2)	n.s.	[90]
	3435	C0 (2)	n.s.	[91]
Efavirenz	3435	C0 (2)	n.s.	[76]
Valacyclovir	1236, 2677, 3435	AUC (1)	n.s.	[93]
Posaconazole	3435	AUC (2)	n.s.	[94]
Voriconazole	2677, 3435	CL (1)	n.s.	[95]
Dicloxacillin	3435	AUC, Cmax (1)	n.s.	[96]
Cloxacillin	1236, 2677, 3435	AUC, Cmax (1)	lower AUC and Cmax in C-G-C homozygous	[97]
CNS				
Phenytoin	3435	C0 (1)	higher C0 in 3435TT	[98]
	3435	C0 (2)	higher C0 in 3435TT	[99]
Carbamazepine	1236, 2677, 3435	C0 (2)	n.s.	[100]
Phenobarbital	2677, 3435	C0, CSF (2)	higher CSF concentration and CSF/C0 ratio in 3435TT	[101]
Risperidone	2677, 3435	C0 (2)	n.s.	[102]
	2677, 3435	C0 (2)	n.s.	[103]
	1236, 2677, 3435	C0 (2)	lower C0 in T-T-T haplotype for active metabolite	[104]
Quetiapine	2677, 3435	transplacental transfer (*ex vivo*)	higher transplacental transfer in 3435TT	[105]
Clozapine	3435	C0 (2)	higher C0 in 3435TT	[106]
Fluvoxamine	3435	C0 (2)	higher C0 in 3435TT	[107]
Paroxetine	61, 2677, 3435	C0 (2)	n.s.	[108]
Citalopram	2677, 3435	C0, CSF (2)	lower C0 and CSF concentration in 2677TT	[109]
Loperamide	3435	AUC, Cmax (1)	n.s.	[111]
	2677, 3435	AUC (1)	higher C0 in 3435TT (trend)	[112]
Methadone	3435	C0 (2)	lower C0 in 3435TT	[113]
	1236, 2677, 3435	d.req. (2)	higher maintenance dose in T-T-T homozygous	[114]
	61, 1199, 1236, 2677, 3435	d.req. (2)	higher maintenance dose in C-G-C homozygous	[115]

(Table 2) Contd…..

Drug	Polymorphism Investigated (exon)	PK parameter	Effect	Refs.
Cardiovascular				
Talinolol	2677, 3435	AUC (1)	n.s.	[116]
	1236, 2677, 3435	AUC, Cmax (1)	n.s.	[117]
Losartan	3435	AUC (1)	n.s.	[118]
Telmisartan	3435	AUC, Cmax, CL (1)	n.s.	[119]
Verapamil	1236, 2677, 3435	C0 (1)	n.s.	[120]
Amlodipine	2677, 3435	AUC, CL (1)	lower AUC and higher CL in T-T homozygous	[121]
Pravastatin	1236, 2677, 3435	AUC, CL (1)	n.s.	[122]
	1236, 2677, 3435	AUC (1)	n.s.	[123]
	2677, 3435	AUC (2)	n.s.	[124]
Fluvastatin	1236, 2677, 3435	AUC, CL (1)	n.s.	[122]
Lovastatin	1236, 2677, 3435	AUC, CL (1)	n.s.	[122]
Rosuvastatin	1236, 2677, 3435	AUC, CL (1)	n.s.	[122]
Simvastatin	1236, 2677, 3435	AUC, CL (1)	higher AUC in T-T-T homozygous	[125]
Atorvastatin	1236, 2677, 3435	AUC, CL (1)	higher AUC in T-T-T homozygous	[125]
Clopidigrel	3435	AUC, Cmax (2)	lower AUC and Cmax in 3435TT	[127]
Miscellaneous				
Fexofenadine	3435	AUC (1)	higher AUC in 3435TT (trend)	[128]
	1236, 2677, 3435	AUC (1)	higher AUC in 3435TT	[129]
Lansoprazole	3435	AUC, Cmax (2)	n.s.	[130]
Budesonide	3435	AUC, CL (1)	n.s.	[131]
Prednisolone	1236, 2677, 3435	AUC, Cmax (2)	n.s.	[132]

PK parameter: (1) single dose, (2) steady-state, d.req.: dose requirement
*When a meta analysis is available, only studies published after the meta analysis are included in this table.
** CYP3A5 expressor status taken into consideration.

important to note that the effect of such a SNP on drug disposition will be drug specific and will have to be analysed carefully for each drug of interest separately (Table **2**). The amino acid change (Ala893Ser/Thr) associated with the 2677G>T/A polymorphism seems to have, on its side, a negligible impact on P-gp function [3]. However, as the three above mentioned SNPs (1236C>T, 2677G>T/A and 3435C>T) are in high linkage disequilibrium, several authors have suggested that the use of haplotype analyses would be of higher relevance for association studies compared to analyses including those SNPs separately, more probably because each SNP has at least an additive effect on protein function/expression [8]. For information, the most frequent *ABCB1* haplotypes are presented in Table **3**, illustrating how the above mentioned 3435T allele, for instance, may belong to different *ABCB1* haplotypes (*2, *13 and *14). Another SNP located in exon 11 (1199G>A, rs2229109) and resulting in an amino acid substitution (Ser400Asn) (Table **1** and **3**) has been shown to alter efflux transport activity of P-gp, probably by modifying substrate specificity [9, 10]. Although less frequent than previously mentioned SNPs (1236C>T, 2677G>T/A and 3435C>T), the 1199G>A polymorphism remains also important to take into account in PK-PG analyses.

In the first study reporting the effect of *ABCB1* 3435C>T polymorphism on intestinal P-gp expression, the authors also assessed the impact of this SNP on the uptake of orally administered P-gp substrates using **digoxin** (DIGO) as a probe drug [5]. As DIGO is mostly excreted as such by the kidney, DIGO PK analysis should mainly reflect its absorption and therefore P-gp activity. In a group of 14 healthy volunteers, Hoffmeyer *et al*. showed that individuals homozygous for this polymorphism (3435TT, n=7) had significantly higher DIGO plasma levels (Cmax) as compared to 3435CC individuals (n=7). The effect on DIGO disposition was in accordance with their initial observation of reduced intestinal P-gp expression at the protein level in 3435TT individuals. Since that time, several studies have investigated the impact of *ABCB1* polymorphisms on DIGO PK parameters in different ethnic groups. In 2005, a first meta-analysis was published on that topic and the overall result, in Caucasian and Japanese subjects taken altogether, suggested that the 3435C>T polymorphism did not seem to have any major influence on exposure levels of DIGO as determined by measurements of area under the plasma concentration/time curve (AUC) between 0 and 4 hours, AUC0-4h or AUC0-24h in Caucasian and Oriental subjects [11]. However, the authors of this meta-analysis mentioned that subjects with 3435CC genotypes were found to have lower DIGO Cmax values compared with subjects bearing 3435TT genotypes, thereby suggesting that the oral availability of DIGO may be lower in subjects with 3435CC geno-

Table 3. Main *ABCB1* Haplotypes (according ref. [3])

Haplotype	61 A>G	IVS4 G>T	IVS9 A>G	1199 G>A	1236 C>T	IVS12 C>T	IVS13 C>T	IVS14 A>G	IVS20 G>A	2677 G>T/A	3435 C>T	CA	AA	AS	ME	PA
*1	A	G	A	G	C	C	C	A	G	G	C	0.150	0.151	0.150	0.200	0.000
*2	A	G	A	G	C	C	C	A	G	G	▽	0.120	0.010	0.016	0.100	0.083
*11	A	G	Ⓖ	G	▽	C	Ⓣ	Ⓖ	G	G	C	0.010	0.020	0.200	0.050	0.166
*13	A	G	Ⓖ	G	▽	C	Ⓣ	Ⓖ	G	[T]	▽	0.320	0.050	0.266	0.350	0.333
*14	[G]	G	Ⓖ	G	▽	C	Ⓣ	Ⓖ	G	[T]	▽	0.075	0.020	0.016	0.000	0.000
*21	A	Ⓣ	A	G	C	Ⓣ	Ⓣ	Ⓖ	G	G	C	0.035	0.080	0.000	0.000	0.000
*24	A	G	A	G	C	C	C	A	G	[A]	C	0.020	0.005	0.066	0.000	0.000
*24A	A	Ⓣ	A	G	C	C	C	A	G	[A]	C	0.000	0.000	0.000	0.000	0.333
*26	A	Ⓣ	A	G	C	C	C	A	Ⓐ	G	C	0.090	0.080	0.050	0.150	0.000
*30	A	G	A	[A]	C	C	C	A	G	G	C	0.010	0.005	0.000	0.000	0.000

Haplotype names were assigned based on evolutionary considerations and assignment of the reference sequence as *ABCB1*1*.
Plain boxes denote the reference sequence; the encircled letters represent, intronic (surrounded by circle), synonymous (surrounded by triangle), and non-synonymous (surrounded by square) segregating sites.
cDNA positions: IVS4 (-25, exon5); IVS9 (-44, exon10); IVS12 (+44, exon12); IVS13 (+24, exon13); IVS14 (+38, exon14); IVS20 (+24, exon20).
Population frequencies are noted. CA: Caucasians; AA: African-Americans; AS: Asian-Americans; ME: Mexican-Americans; PA: Pacific Islanders.

type [11]. It is very interesting to note that the effect of this 3435C>T polymorphism was actually different according to the ethnic group investigated. Indeed, Caucasians volunteers bearing the 3435TT genotype had on average higher Cmax or AUC0-4h or AUC0-24h [12, 13] whereas Japanese volunteers showed the opposite trend [14, 15]. More recent studies, published after that meta-analysis, have confirmed higher DIGO levels (Cmax, AUC0-24h) in Caucasians volunteers bearing the 3435TT genotype [16, 17]. Accordingly, a recent study on 195 Caucasians patients receiving DIGO as part of their chronic therapy for congestive heart failure has shown that DIGO serum levels (trough level C0 measured for therapeutic drug monitoring purposes) were significantly higher in the group of patients bearing the 3435TT genotype (0.18-0.21 µg/L per additional T allele) and more particularly the 1236T-2677T-3435T haplotype [18]. However, another study also performed in Caucasians patients treated with DIGO (n=77) did not show a similar trend probably, according to the authors themselves, because of the influence of P-gp inhibitor co-administration frequently encountered in daily clinical practice [19]. Altogether, existing literature data indicate that DIGO PK parameters are clearly influenced by *ABCB1* polymorphisms in a different way according to the ethnicity and that haplotype analysis would be useful to better understand such discrepancies. However, although statistically confirmed, the clinical relevance of these polymorphisms on DIGO PK still remains to be determined mainly in view of the co-administration of P-gp inhibitors in routine clinical practice.

Several immunosuppressive drugs, largely used to prevent graft rejection after solid organ transplantation, are well known substrates of ABCB1. Among them, calcineurin inhibitors (CNIs), mainly represented by **cyclosporine** (CsA) and tacrolimus (Tac), prevent cellular rejection through the selective inhibition of interleukin-2 production by T-cells.

The effect of the 3435C>T polymorphism on CsA PK parameters has been recently reviewed in a meta-analysis mainly based on results obtained from patients treated with CsA [20]. The main CsA PK parameters taken into account in this meta-analysis were CsA AUC0-4, AUC0-12 (twice a day administration drug), CL/F, Cmax and C0 (trough level). While the authors found no significant effect of the 3435C>T polymorphism on CsA AUC0-4 [8, 21-23], a significant influence of this polymorphism was observed on CsA AUC0-12, with higher values for patients bearing at least one 3435T allele [8, 21, 24]. A possible explanation for the discrepancy observed between AUC0-4 and AUC0-12 is the fact that P-gp is expected to influence the PK of its substrates, such as CsA, by limiting their absorption but also by promoting their excretion (P-gp is expressed at the biliary and renal tubular level) and that the information obtained from CsA AUC0-4 would be more influenced by the absorption phase than the potentially affected elimination phase. Contrary to DIGO, this meta-analysis did not find any evidence to support the influence of 3435C>T polymorphism on CsA Cmax [8, 21, 25, 26]. This may suggest that the effect of 3435C>T polymorphism on the oral availability of CsA is limited. This observation is in accordance with the findings mentioned above that CsA AUC0-4 seems not to be influenced by different C3435T genotypes, thereby suggesting that the 3435C>T polymorphism may have a greater influence on excretion (and possibly distribution) than absorption of CsA [20]. Finally, the meta-analysis showed a significant impact of the 3435C>T polymorphism on trough CsA concentrations (C0), but only after exclusion of two studies including Asian patients [21, 22, 24, 27-30]. As with DIGO, these findings suggest ethnic differences in genotype-phenotype relationships involving ABCB1. Interestingly, it was recently confirmed that ABCB1 polymorphism was associated with differences in CsA PK but only during the first week post-transplant [23]. Such a finding could be partly explained by the inhibition of P-gp by CsA itself. Taken together, all results indicate that the influence of the 3435C>T polymorphism on whole blood CsA PK parameters, although significant, at least for some of them (AUC0-12, C0), is probably of a magnitude unlikely to be relevant for clinical practice. It should be noted, however,

that whole blood CsA concentrations, depending on individual pharmacokinetics and pharmacogenetics (PG), do not necessarily reflect intracellular concentration and hence the ability to interact with the ultimate target, calcineurin. Since T-cells are the pharmacodynamic target of CNIs, genetic studies investigating CNIs concentrations within this cell population would be more relevant from a clinical point of view. In the case of CsA, Crettol *et al*. recently showed that whole blood concentrations only partially reflected the highly variable intracellular (or intralymphocyte) concentrations, and that for a given CsA blood concentration, intracellular concentration varied over a 10-fold factor among patients [31]. More interestingly, they showed that CsA intracellular concentrations, and to a lesser extent intracellular *versus* blood concentration ratios (an accumulation index), were determined by genetic polymorphisms in *ABCB1*, also expressed at the cellular membrane of T-cells. The *ABCB1 1199A* allele (1199G>A polymorphism) was associated with lower intracellular concentrations, probably caused by a higher P-gp activity towards CsA, whereas the *3435T* allele (3435C>T polymorphism) led to higher intracellular concentrations, most probably due to a lower P-gp activity towards the drug, as observed previously for CsA whole blood PK [31]. Importantly, the authors also showed that *ABCB1* polymorphisms influenced CsA intracellular concentrations to a significantly higher extent than CsA blood concentrations, with therefore a greater potential in term of clinical impact. Such studies are important in that they provide physicians with genetic determinants of drug concentration at the pharmacodynamic target, opening new therapeutic perspectives. Indeed, integrating such data (with others still to be elucidated) in a physiologically-based pharmacokinetic (PBPK) model might, for instance, facilitate simulation of intracellular versus blood concentration relationships and contribute to extrapolate intracellular concentrations from whole blood measurements, performed routinely in all laboratories involved in TDM activities. Such an approach could therefore offer a useful alternative to the laborious methods to measure intracellular concentrations (not performed routinely) in that individualised whole blood or plasma target concentrations could be proposed, according to the genotype each patient, to ultimately reach a similar effective intracellular concentration. Although out of the scope of this review, and considering that ABCB1 is also expressed by kidney tubular cells, differences in *ABCB1* genotype (3435C>T polymorphism) have been demonstrated to significantly impact nephrotoxocity, a major CsA side effect, most probably by influencing CsA intrarenal concentrations [32]. The influence of *ABCB1* polymorphisms on CsA intracellular concentrations, contrary to whole blood concentrations, remains therefore a topic with a great potential clinical interest and further studies should assess its real PK and pharmacodynamic (PD) impact.

Tacrolimus (Tac) is another CNI largely used in solid organ transplantation. It is an ABCB1 substrate and polymorphisms in the gene coding for this ABC transporter could also theoretically influence Tac PK parameters. However, it should be kept in mind that Tac whole blood PK parameters are largely influenced by genetic polymorphisms in CYP3A5, the main CYP isoform involved in Tac biotransformation [29] and that this genetic association has now to be considered as a important confounding factor when assessing any possible association between *ABCB1* polymorphisms and Tac whole blood PK parameters. Indeed, it is now well established that whole blood Tac concentrations (and dose requirements) are strongly associated with the CYP3A5 expressor status, which is itself genetically determined [33]. Numerous reports have confirmed that patients expressing CYP3A5 (around 20% of the Caucasian population) have a lower Tac concentration to dose ratio when compared with nonexpressors in kidney but also in liver, lung and heart transplant recipients [34]. In a cohort of kidney transplant recipients, Tac dose-adjusted trough concentrations were 5.8-fold lower in CYP3A5 expressors than in CYP3A5 nonexpressors and there was a 2.3-fold difference to maintain target blood concentrations between both groups [29]. With this in mind and taking into consideration the confounding CYP3A5 expressor status, the association between Tac PK parameters and *ABCB1* polymorphisms is still a matter of debate [34]. Indeed, most of the studies failed to demonstrate any association between *ABCB1* polymorphisms and Tac dose requirements or dose-adjuted trough blood concentrations [28, 29, 35-44] whereas only a few reports suggested a significant, but often weak, relationship between *ABCB1* polymorphisms and both Tac PK parameters in heart [45], lung [46], liver [47] or kidney [48-53] transplant recipients with *3435T* carriers always requiring lower doses to reach the target blood concentrations of Tac. Confounding factors, including the CYP3A5 expressor status, may, of course, play a major role in these conflicting results. In this respect, an independent effect of *ABCB1* polymorphisms should be better assessed separately in CYP3A5 nonexpressors and CYP3A5 expressors. Only a few studies have been conducted with such a design and they mainly showed that the association between *ABCB1* polymorphisms and Tac PK parameters was either not significant [2, 54] or, even more interestingly, that it disappeared after sub-classification according to CYP3A5 expressor status in a large cohort of stable renal transplant patients (n=206) with high statistical power [55]. Only one study performed on 91 lung transplant recipients suggested that ABCB1 haplotypes derived from the three common polymorphisms (exons 12, 21 and 26) was associated with Tac dose requirement after eliminating the confounding CYP3A5 genotype [56]. Clearly, all these studies indicate that Tac whole blood PK parameters are mainly influenced by the CYP3A5 expressor status and that *ABCB1* polymorphisms only play a minor role, if any. Furthermore, as for CsA, whole blood Tac concentrations do not necessarily reflect their intracellular concentration and hence their efficacy or toxicity. In a pilot study, Tac concentrations in hepatic tissue have been shown to better correlate with acute rejection score than whole blood concentrations in adult liver-transplanted recipients from whom protocol biopsies were obtained [57]. Interestingly, in the same study population, a significant relationship was observed between intrahepatic Tac concentrations and *ABCB1* polymorphisms, whereas no correlation was seen when Tac whole blood concentrations were considered independently [58]. As for CsA, this latter report suggests that *ABCB1* genotypes may influence Tac biliary excretion and/or tissue distribution, but further studies are still needed to confirm these findings. Preliminary, yet unpublished, data from our group seeking to determine Tac concentrations within lymphocytes suggest similar conclusions. The *1199A*

(1199G>A polymorphism) and *3435T* (3435C>T polymerphism) alleles were significantly associated with increased intracellular concentrations probably because of a lower P-gp activity towards Tac and therefore with a potentially higher immunosuppressive activity. As for CsA, the latter studies suggest that the influence of *ABCB1* polymorphisms on Tac intracellular concentrations is probably of higher clinical relevance compared to their influence on whole blood concentrations.

Sirolimus (SRL) is another immunosuppressive drug largely used in solid organ transplantation and belonging to the *mammalian target of rapamycin* (mTOR) inhibitor group, a distinct pharmacological class compared to CNIs, that inhibits the response to interleukin-2 and thereby blocks the activation of T- and B-cells. SRL is also a substrate of the ABCB1 transporter but *ABCB1* polymorphisms seem to have no impact on SRL whole blood PK parameters [59-62]. To the best of my knowledge, data on the influence of *ABCB1* polymorphisms on SRL intracellular concentrations are not available.

Several anti-cancer drugs are also well known substrates of ABCB1 and the impact of *ABCB1* polymorphisms, mainly 3435C>T polymorphism, on their PK parameters has been investigated for some of them.

In the group of microtubule inhibitors, **docetaxel** (DOC) and **paclitaxel** (PAC) PK parameters have been largely investigated in relation to *ABCB1* genetic status. The DOC AUC and CL have been reported, in most cases, to be unaffected by *ABCB1* SNPs [63, 64] but one study in 92 Caucasians patients reported a significantly decreased DOC CL only in *ABCB1* 1236TT homozygous (-25%) and no influence of 2677G>T/A and 3435C>T polymorphisms [65]. A weak association between PK parameters and *ABCB1* genetic status has been also described for PAC [66-68]. For example, in the study of Nakajima *et al*., patients possessing the 3435T allele had a significantly higher AUC of 3'-p-hydroxyPAC (the main PAC metabolite) compared to those possessing the 3435C allele. However, adverse effects of PAC (mainly leucopenia) were not related to 3'-p-hydroxyPAC AUC [66]. Therefore, even if *ABCB1* genetic status has the potential to alter the PK of the main PAC metabolite, the influence of *ABCB1* genotype on intracellular PAC and 3'-p-hydroxyPAC concentrations still remains to be determined. For **vincristine** (VIN), another anti-cancer drug belonging to the group of microtubule inhibitors, neither *ABCB1* genotype nor *ABCB1* haplotype influenced PK parameters such as AUC and CL [69].

In the group of topoisomerase 1 inhibitors, **irinotecan** (CPT-11) and its active metabolite (SN-38) PK parameters have been shown to be influenced by *ABCB1* haplotype [70]. In this latter study, the authors showed that the renal CL of both CPT-11 and SN-38 was reduced in patients belonging to the *ABCB1* haplotype 1236T-2677T-3435T, suggesting that P-gp expressed in the proximal tubules may play a substantial role in the renal excretion of CPT-11 and SN-38 [70]. Unexpectedly, another study, also performed in patients from Asian origin, has shown that the *ABCB1* 2677G-3435C haplotype was associated with a higher SN-38 AUC without any influence on CPT-11 AUC [71]. The reasons for these discrepancies are not yet clear but it could be concluded that the impact of *ABCB1* genotype on CPT-11 PK parameters, if present, seems very limited and only present after haplotype analysis.

In the group of tyrosine kinase inhibitors, **imatinib** (IMA) has been investigated as a P-gp substrate. Although a preliminary study did not shown an influence of *ABCB1* genotype on IMA PK parameters [72], two more recent studies found an association [73, 74]. In the first study, Gurney *et al*. showed that the reduction in IMA CL between day 1 and steady-state was associated with *ABCB1* genotype, being less apparent in homozygous 1236TT-2677TT-3435TT, probably due to a genotype-specific influence of IMA on elimination [73]. In the second study, Dulucq *et al*. showed that patients with 1236TT genotype had higher IMA concentrations and, accordingly, that the 1236C-2677G-3435C haplotype was statistically associated to less frequent major molecular response demonstrating a potential usefulness of these SNPs in the identification of who may or may not respond optimally to IMA [74].

Studies have been also conducted with **doxorubicin** (DOX). In a recent paper on Asian breast cancer patients, the authors showed that patients homozygous for the 1236C-2677G-3435C haplotype had significantly lower DOX exposure levels compared to the patients carrying the CT-GT-CT ($p=0.02$) or TT-TT-TT ($p=0.03$) genotypes. Accordingly, significantly increased DOX CL was also observed in patients harbouring CC-GG-CC genotype when compared to patients harbouring the CT-GT-CT genotype [75].

In summary for anti-cancer drugs, *ABCB1* genotype may alter IMA and DOX PK parameters but studies exploring the impact of *ABCB1* SNPs on intracellular drug concentrations (and adverse effects) are still needed.

Among anti-infective drugs, the human immunodeficiency virus (HIV) protease inhibitors (PIs) are well known substrates of P-gp and several studies investigating the impact of *ABCB1* genotype and/or haplotype on their PK parameters have been published since early 2000. **Lopinavir** (LPV) is the most widely used PI (in association with low doses of ritonavir, RTV, used as a booster). To the best of my knowledge, all studies that have investigated the impact of *ABCB1* genotype on LPV trough concentrations (C0) in HIV treated patients have led to negative results [76-78]. Similar negative results have been obtained for **saquinavir** (SQV), another PI, based on studies performed in volunteers and analysing PK parameters such as SQV AUC and CL [79-81]. In the case of **indinavir** (IDV), although negative results have been obtained in large cohort of patients when considering IDV C0 [82], 3435C>T polymorphism has been associated with absorption constant rate, Vd and CL [83, 84], suggesting that patients with 3435CC genotype would have reduced IDV absorption after oral intake. The situation seems somewhat different for **atazanavir** (ATZ), a more recent PI for which a significant association has been observed between trough concentrations (C0) and *ABCB1* genotype. Indeed, it has been reported that plasma trough concentrations of ATZ were significantly higher (about 2.5-fold) in HIV treated patients with 3435CC genotype compared to those with CT or TT genotype [85, 86]. It is interesting to note that this effect of 3435C>T polymorphism on ATZ C0 is at the opposite of the one observed with DIGO. This observation could be explained either by the fact that 3435C>T SNP alters substrate specificity as suggested by

Kimchi-Sarfaty *et al*. [7] or by a different ATZ redistribution in peripheral blood mononuclear cells and in particular in T lymphocytes, the target of antiretroviral therapy. Unfortunately, no data are available in relation to ATZ intracellular concentrations and *ABCB1* genotype. This association between *ABCB1* genotype and ATZ C0 was not confirmed in the study of Ma *et al*. [77]. The case of **nelfinavir** (NFV) is interesting in the way that intracellular data are available. When considering only NFV plasma concentrations (C0), the effect of *ABCB1* genotype is not clear. While one study found no association between NFV C0 and *ABCB1* genotype [87], another study in children found that NFV C0 was higher in 3435CT heterozygous compared to 3435CC homozygous but values observed for 3435TT were not different from those observed for 3435CC patients [88]. As for immunosuppressive drugs (e.g. CsA, Tac), the ABCB1 efflux pump activity may decrease intracellular drug concentration, thus reducing the amount of drug at the site of action (in this case, T lymphocytes). Based on the measurement of NFV in PBMCs of HIV infected patients, Colombo *et al*. were the first to demonstrate the existence of a nice relationship between NFV intracellular PK and 3435C>T polymorphism with intracellular AUC ratios of 2.1, 1.4 and 1 for *ABCB1* 3435TT, CT and CC patients, respectively, without improvement after haplotype analysis [89]. Based on these results, the presence of the 3435T allele should be associated with a reduced ABCB1 efflux activity towards NFV as observed initially with DIGO [5].

The non-nucleoside reverse transcriptase inhibitors (NNRTIs) are another category of antiretrovirals used in combination with PIs to treat HIV infection in humans. **Nevirapine** (NVP) and **efavirenz** (EFV) are the main substances belonging to this pharmacological class. The studies assessing the association between *ABCB1* genotype and either NVP C0 [90, 91] or EFV C0 [76] have led to negative results. These results are not surprising because NNRTIs, contrary to PIs, are poor P-gp substrates [92].

The PK parameters of the antiviral **valacyclovir** (VLC) do not seem associated with *ABCB1* genotype [93].

Antifungals belonging to the triazole class are also P-gp substrates but neither **posaconazole** AUC [94] nor **voriconazole** CL [95] seem to be associated with *ABCB1* genotype.

Among antibiotics, the *ABCB1* genotype did not influence **dicloxacillin** PK parameters (AUC, Cmax) [96] whereas **cloxacillin** PK parameters were only influenced by *ABCB1* haplotype (and not genotype) with patients homozygous for the 1236C-2677G-3435C haplotype having a significantly lower Cmax (2.3-fold), AUC (1.6-fold) and urinary excretion (2.2-fold) of cloxacillin compared with those carrying the homozygous for the T-T-T haplotype [97].

Many drugs exert their pharmacological activity at the central nervous system (CNS) level. As P-gp is also expressed at the endothelial cells of the blood-brain barrier (BBB), its efflux activity may limit the uptake of those drugs within the CNS and therefore reduce their pharmacological activity and/or toxicity. In this respect, PK parameters based on cerebrospinal fluid (CSF) concentrations would be more informative in comparison to PK parameters obtained from plasma or blood concentrations. Furthermore, the calculation of CSF/plasma or blood concentration ratios may be useful as an index of drug penetration across the BBB in order to assess the potential impact of *ABCB1* genotype on P-gp activity *in vivo*.

Antiepileptic drugs certainly belong to this category. The **phenytoin** (DPH) plasma trough concentrations C0 have been associated with 3435C>T polymorphism; indeed, patients or volunteers of Caucasian origin and bearing the 3435TT genotype had significantly higher DPH C0 compared to those bearing at least one 3435C allele (CT and CC) [98, 99]. No effect of *ABCB1* genotype was observed in epileptics of Asian origin on the plasma PK parameters of **carbamazepine** (CBZ) [100]. However, the latter authors observed that *ABCB1* genotype influenced the CBZ responsiveness without significant changes in the CBZ plasma concentrations, suggesting the potential interest for CBZ measurements in CSF. Such an analysis has been performed for **phenobarbital** (PB) in Caucasian epileptic patients [101]. In this study, the authors clearly showed that patients with 3435TT genotype had significantly higher PB CSF concentrations and CSF/plasma ratios (1.5-fold compared with 3435CC for both parameters) without any changes in PB plasma concentrations. Furthermore, the seizure frequency was significantly reduced when PB CSF concentrations were increased and when patients were bearing 3435T alleles [101].

Antipsychotic and antidepressant drugs have also to cross the BBB to exert their pharmacological activity. Two studies conducted in Asian schizophrenia patients treated by **risperidone** (RIS) showed that plasma trough C0 was not associated with *ABCB1* genotype [102, 103]. A more recent study involving schizophrenia patients from Caucasian origin confirmed the observation on Asian patients but showed an effect of *ABCB1* haplotype on the plasma concentration of 9-hydroxyrisperidone, the active metabolite of RIS, with lower values in patients homozygous for the 1236T-2677T-3435T haplotype [104]. The RIS antipsychotic effect is assumed to be related to the active moiety, that is, the sum of RIS and 9-hydroxyrisperidone, which was also significantly lower in patients homozygous for the T-T-T haplotype [104]. **Quetiapine** (QTP) is another antipsychotic for which a higher placental transfer has been reported in association with the presence of the 3435T allele [105]. Patients carriers of the ABCB1 3435TT genotype have been shown to have 1.6-fold higher **clozapine** (CLOZ) plasma concentrations than non carriers [106]. Unfortunately, no data are available on RIS, QTP and CLOZ concentrations in CSF in relation to *ABCB1* genotype.

In Asian psychiatric patients, it has been shown that the plasma PK of the antidepressant **fluvoxamine** (FLV) was associated with *ABCB1* genotype, with higher FLV C0 in 3435TT patients (2-fold compared to 3435CC), but only for the higher dosages (200 mg/day) [107]. In Caucasian patients treated by antidepressant drugs, no effect of *ABCB1* genotype was observed on **paroxetine** (PRX) plasma concentrations [108] while an opposite result was observed after **citalopram** (CIT) use with lower CIT C0 values in 2677TT patients (1.5-fold compared to GG/GT) [109]. Interestingly for the latter study, the 2677TT genotype was also associated with lower CIT CSF concentrations (1.33-fold compared to GG/GT), and a lower treatment response,

suggesting that this particular genotype was associated with an increased activity of ABCB1 towards CIT [109]. Unfortunately, the haplotype analysis was not reported in this study.

To account for the highly variable PK of antipsychotic and antidepressant drugs, besides the potential impact of *ABCB1* genotype, as discussed above, it must be kept in mind that *CYP2D6* genotype plays a major role so that guidelines for dosage adjustments according to *CYP2D6* status are already available for these drugs [110]. However, for a given plasma concentration, *ABCB1* status could still explain a part of the variability in CSF concentration, as shown previously with CIT, and ultimately in therapeutic response.

Opioids and analogues also exert their pharmacological activity after crossing the BBB. The PK of the anti-diarrhoea drug **loperamide** (LPM) has been analysed in relation to *ABCB1* genotype with no clear results in Caucasians volunteers. While one study showed no association between 3435C>T polymorphism (or *ABCB1*1* vs *ABCB1*13* haplotype carriers) and LPM plasma concentration [111], another study showed only a non significant trend towards higher plasma LPM in 3435TT carriers suggesting a reduced activity of ABCB1 towards LPM for 3435T allele [112]. No data about LPM CSF concentrations were available in both studies. The effect of *ABCB1* genotype seems more pronounced on **methadone** (MTD) PK. In a study including 245 Caucasians patients undergoing MTD maintenance treatment, Crettol *et al*. showed that 3435TT carriers presented lower (1.26-fold) trough MTD plasma concentrations compared to 3435CC carriers [113]. In accordance with those results, it has been shown that patients bearing the 1236TT-2677TT-3435TT genotype had an approximately 5-fold chance of requiring a higher MTD maintenance dose [114] most probably due to a reduced uptake of MTD in the CNS, illustrating the potential drug specific effect of *ABCB1* polymorphisms [7]. It should be mentioned that a study has found opposite results also in Caucasian patients [115] and that data about MTD CSF concentrations were not available in those studies.

In the cardiovascular field, several drugs are also ABCB1 substrates. No effect of *ABCB1* genotype on blood PK parameters was observed for **talinolol** [116, 117], **losartan** [118], **telmisartan** [119] and **verapamil** [120]. It has been shown, however, that **amlodipine** (ALD) PK parameters were affected by *ABCB1* haplotype in Asians volunteers [121]. In this study, lower ALD AUC (1.48-fold) and higher ALD CL (1.52-fold) were observed in volunteers bearing the 2677TT-3435TT genotype than in those with 2677GG-3435CC genotype, suggesting an increased activity of ABCB1 towards ALD for the 2677T-3435T haplotype. The *ABCB1* genotype seems to have different effects on the PK of cholesterol-lowering HMG-CoA reductase inhibitors statins. While the PKs of **fluvastatin** [122], **pravastatin** [122-124], **lovastatin** [122] and **rosuvastatin** [122] have been shown to be unaffected by the *ABCB1* genotype, a modest increase in AUC for **simvastatin** acid (1.6-fold) and **atorvastatin** (1.55-fold) has been observed in volunteers homozygous for the 1236T-2677T-3435T haplotype compared with those homozygous for the 1236C-2677G-3435C haplotype [125]. However, it must be stressed that the plasma concentration of statins, except fluvastatin, is highly dependent of a SNP in the influx transporter OATP1B1 [126] which has to be considered as a major confounding factor when analysing the impact of *ABCB1* genotype. The PK of the antiplatelet prodrug **clopidogrel** (CPG) has been also associated with *ABCB1* genotype. In Caucasian patients with coronary artery disease who underwent percutaneous coronary intervention, CPG Cmax and AUC were significantly lower (3.73- and 4.69-fold, respectively) in subjects homozygous for the 3435T allele compared with subjects with the 3435CT and CC genotype suggesting that CPG absorption (and thereby active metabolite formation) is decreased in presence of the 3435T allele [127].

Among miscellaneous drugs known to be ABCB1 substrate, **fexofenadine** (FEX) PK has been analysed in relation to *ABCB1* genotype. In Caucasian volunteers, a non-statistically significant trend towards higher FEX AUC was observed in subjects bearing the 3435TT genotype [128] while similar results became significant in Asians volunteers [129]. Interestingly, in the latter study, the authors showed that the *ABCB1* genotype 2677AA-3435CC was characterised by the lower FEX plasma concentrations compared to other main genotype groups (2677GG-3435CC or 2677TT-3435TT) highlighting the interest of haplotype analysis in different ethnic populations. No effect of *ABCB1* genotype was observed on the PK of **lansoprazole** [130], **budesonide** [131] and **prednisolone** [132].

ABCC2

ABCC2 (also known as *multidrug resistance protein 2*, MRP2) is an ATP-binding cassette transporter mainly responsible for the biliary excretion of numerous organic anions. For this reason, it was originally referred to as *canalicular multispecific organic anion transporter* (cMOAT). Its main physiological substrate is bilirubin glucuronide and a genetic defect in bilirubin glucuronide excretion due to *ABCC2* mutation leads to the Dubin-Johnson syndrome (DJS). Several mutations in *ABCC2* have been therefore described in patients suffering from the DJS. Besides bilirubin glucuronide, ABCC2 is also responsible for the active efflux of many drugs, influencing, as for ABCB1, biliary and renal excretion, intestinal absorption and tissue distribution [133]. Interestingly, ABCC2 is also expressed on the membrane of PBMCs, particularly in CD4+ T cells [134]. In addition to mutations responsible for the DJS, several SNPs have been also reported for *ABCC2,* some of which affecting transporter expression and/or function (see ABCC2 on http://www.pharmgkb.org/search/) (Table **4**). Four among those SNPs, namely -24C>T (rs717620), 1249G>A (rs2273697), 3972C>T (rs3740066) and 4544G>A (rs8187710), located in 5'-UTR, exons 10, 28 and 32, respectively, have been studied extensively mainly because of their high allele frequency in humans. The 1249G>A and 4544G>A polymorphisms result in an amino acid substitution (Val417Ile and Cys1515Tyr) whereas the 3972C>T is a synonymous SNP (Ile1324Ile). Furthermore a linkage disequilibrium between -24C>T and 3972C>T has been observed [133]. An *in vitro* analysis has shown that the *ABCC2* -24C>T polymorphism was associated with a 20% reduction in transcriptional activity in HepG2 cells, and

Table 4. Important Polymorphisms (SNPs) in *ABCC2* Gene Selected in PharmGKB Database (http://www.pharmgkb.org/search/)

Position	Nucleotide Change	Effect[a]	NCBI dbSNP	Allelic Frequency
5'-flanking	-1549G>A	-	rs1885301	Ca: 0.43 As: 0.10 Af: 0.49
5'-flanking	-1019A>G	-	rs2804402	Ca: 0.43 As: 0.15 Af: 0.37
5'-UTR[c]	-24C>T*	-	rs717620	Ca: 0.19 As: 0.10 Af: 0.06
Exon 10	1249G>A	Val417Ile	rs2273697	Ca: 0.17 As: 0.10 Af: 0.17
Exon 10	1446C>G	synonymous		
Exon 18	2366C>T	Ser789Phe	rs56220353	Ca: 0.00 As: 0.02 Af: 0.00
Exon 25	3542G>T	Arg1181Leu	rs8187692	Ca: 0.00 As: 0.00 Af: 0.09
Exon 25	3563T>A**	Val1188Glu	rs17222723	Ca: 0.08 As: 0.00 Af: 0.07
Exon 28	3972C>T*	synonymous	rs3740066	Ca: 0.38 As: 0.20 Af: 0.27
Exon 31	4348G>A	Ala1450Thr	rs56296335	Ca: 0.00 As: 0.02 Af: 0.00
Exon 32	4544G>A**	Cys1515Tyr	rs8187710	Ca: 0.08 As: 0.00 Af: 0.20

NCBI: National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/projects/SNP/)
[a]number represents amino-acid codon
[b]based on information found in http://www.pharmgkb.org/search/
Ca: Caucasian, As: Asian, Af: African
[c]UTR: untranslated region
* linkage disequilibrium between -24C>T and 3972C>T
** linkage disequilibrium between 3563T>A and 4544G>A

lower levels of mRNA were detected in kidney tissues of carriers of this SNP [135].

Among immunosuppressive drugs used in transplantation, **mycophenolic acid** (MPA) PK has been largely investigated in relation to *ABCC2* genotype. Briefly, MPA is glucuronidated by uridine diphosphate glucuronosyltransferases (UGTs, mainly UGT1A9 isoform) to its pharmacologically inactive 7-O-glucuronide metabolite (MPAG). MPA is also glucuronidated, to a lesser extent (about 5%) and mainly by UGT2B7 isoform, to acyl-MPAG (AcMPAG) which is believed to have immunosuppressive but also toxic properties (diarrhoea). MPAG (but also MPA and AcMPAG) is then excreted into the bile via ABCC2, which is essential for enterohepatic (re)circulation (EHC) of MPA(G). Moreover, MPA AUC ratio (AUC6-12/AUC0-12) has been proposed by some authors as an estimate of EHC of MPA. In a preliminary study including Caucasian renal allograft recipients in steady state conditions, Naesens *et al*. have shown that *ABCC2* -24C>T polymorphism (and also 3972C>T in linkage disequilibrium) was associated with higher MPA trough levels (C0) due to a lower MPA CL [136]. Similarly, MPA CL was also significantly lower (-20%) in Japanese renal transplant recipients bearing the *ABCC2* -24T allele [137] whereas no effect of *ABCC2* genotype was observed in a smaller cohort of 40 Caucasian renal transplant patients [138]. These observations could be explained by a lower ABCC2 activity towards MPA in -24T carriers. Interestingly, using an analytical method validated to measure AcMPAG and MPAG in addition to MPA, Lévesque *et al*. showed that *ABCC2* -24C>T polymorphism was also associated with a 25% increase in AcMPAG AUC after single administration of the drug in Caucasian volunteers, suggesting a reduced ABCC2 activity towards AcMPAG and maybe a reduced intestinal toxicity in patients bearing the -24T allele [139]. A similar effect on AcMPAG AUC was observed in Chinese renal transplant recipients but this time with the *ABCC2* 1249G>A polymorphism and patients carrying the heterozygous genotype *ABCC2* 1249GA exhibited a higher AcMPAG AUC6-12 compared to the wild-type genotype [140]. All those studies indicate that *ABCC2* genotype influence MPA and MPA metabolites PK parameters. Contrary to MPA, *ABCC2* genotype seems to have a negligible impact on **tacolimus** (Tac) and **sirolimus** (SRL) trough concentrations in renal transplant recipients [61].

Among anticancer drugs, **methotrexate** (METO) and **irinotecan** (CPT-11) have been investigated in relation to *ABCC2* genotype. In a group of 44 Caucasian paediatric patients with acute lymphoblastic leukemia, Rau *et al*. showed that the mean plasma METO AUC from 36 to 48 hours after the start of the infusion was significantly 2-fold higher in female patients carrying at least one *ABCC2* -24T allele as compared with all other patients [141]. Similar results have been published with CPT-11. In a cohort of 85 Caucasian cancer patients, a higher CPT-11 AUC has been observed in those bearing the *ABCC2* -24T allele and, in this study, more than 40% of the variation in CPT-11 AUC could be explained by five independent variables among which *ABCC2* -24C>T, age and CPT-11 dose [142]. Similarly, in a group of 67 Asian cancer patients, *ABCC2* haplotype I (including the -24C allele, see Table **5**) and haplotype IV (including the 1249 A allele) were respectively associated with lower SN-38 (CPT-11 active metabolite) and lower CPT-11 AUCs [143]. In a cohort of 167 Caucasian cancer patients, although no effect was observed for individuals SNPs, an *ABCC2* haplotype (including the 1249G allele) has been associated with lower CPT-11 CL (28.3 vs 31.6 L/h) and with a significant reduction of severe diarrhoea, maybe as a consequence of reduced hepatobiliary secretion of CPT-11 itself [144]. All those results suggest that *ABCC2* genotype, particularly -24C>T and 1249G>A polymorphisms, may be respectively associated with reduced and increased

Table 5. Main *ABCC2* Haplotypes

Haplotype	-24C>T	1249G>A	1446C>G	3563T>A	3972C>T	4544G>A	*
I	C	G	C	T	C	G	0.31
II	T	G	C	T	T	G	0.23
III	C	G	C	T	T	G	0.16
IV	C	A	C	T	C	G	0.19
V	C	G	C	A	C	A	0.04

* haplotype frequency according Lévesque *et al.* [139].

excretion of some anti-cancer drugs including METO and CPT-11, with potential clinical effects on toxicity, as discussed previously [144], or on efficacy (higher response rate in *ABCC2* -24TT patients) [71]. However, Zamboni *et al*. did not find any effect of *ABCC2* -24C>T polymorphism on the disposition of 9-nitrocamptothecin and its 9-aminocamptothecin metabolite [145], illustrating once more the difficulties of *in vivo* PK-PG analyses.

As discussed for ABCB1 and antiretrovirals, the ABCC2 efflux pump activity may decrease intracellular drug concentration, thus reducing the amount of drug at the site of action (in this case T lymphocyte). While no effect of *ABCC2* genotype was observed on **nelfinavir** [89] or **tenofovir** [146] intracellular concentrations, a significant association has been observed between *ABCC2* 4544G>A polymorphism and **lopinavir** (LPV) intracellular concentration suggesting that *ABCC2* 4544A allele (haplotype V, linked with 3563A allele) could be associated with a reduced activity of ABCC2 towards LPV [147].

In relation to cholesterol-lowering HMG-CoA reductase inhibitors, no effect of the most frequently assessed *ABCC2* SNPs was observed on **pravastatin** [123, 148] or **simvastatin** [149] PK parameters. However, after taking into consideration the main genetic confounding factor in statins PK (i.e. the OATPB1 genotype), Niemi *et al*. have shown that volunteers heterozygous for the *ABCC2* 1446C>G polymorphism presented AUC0-12 and Cmax of pravastastin respectively 67 and 68% lower than in those with the 1446CC genotype [150]. As they also demonstrated that ABCC2 mRNA expression was 95% higher in livers with the 1446CG genotype (n=7) than in those with 1446CC genotype (n=86), the authors suggested that *ABCC2* 1446C>G polymorphism was associated with reduced systemic exposure to pravastatin as a consequence of increased ABCC2 expression.

As observed with CPT-11, the variant *ABCC2* 1249G>A has been associated with lower oral bioavailability and increased residual CL of intravenous **talinolol** suggesting that Val417Ile substitution is associated with higher activity of the intestinal transporter [151]. Finally, in 12 Japanese renal transplant recipients, those with the *ABCC2* -24CT genotype showed significantly higher Cmax of **telmisartan** compared to those with the -24CC genotype (96.8 *vs*. 57.4 ng/ml, respectively) [152].

In conclusion, genetic variability in the *ABCB1* and/or *ABCC2* genes may potentially influence drug disposition, probably by altering protein expression and/or function. However, in most cases, the impact of *ABCB1* and/or *ABCC2* SNPs on blood PK parameters remains very controversial (because relatively weak) and difficult to interpret for several potential reasons.

First, it has been suggested that it was probably more relevant to emphasis on haplotypes rather than on isolated SNPs and to take into account the haplotype variability in different racial backgrounds. Indeed, each SNP belonging to a particular haplotype may have at least an additive effect on protein function/expression. However, well designed studies involving genetic association with *ABCB1* (according to Table **3** criteria) or *ABCC2* haplotypes and drug PK parameters are still quite rare. Secondly, a simultaneous effect of SNPs including other transporters and/or biotransformation enzymes may hide the potential effects of *ABCB1* and/or *ABCC2* SNPs as illustrated previously with cholesterol-lowering statins, immunosuppressants or antipsychotics/ antidepressants and OATP1B1, CYP3A5 or CYP2D6, respectively. To overcome this problem, further studies will have to combine several SNPs including different proteins and of course to analyse larger cohorts of patients or volunteers with sufficient study power. Thirdly, it must be reminded that ABCB1 and ABCC2 are specifically expressed in different tissues and that a same SNP may simultaneously affect different processes, such as absorption, elimination, and tissue distribution, as illustrated in Fig. (**1**). The resulting effect on drug concentrations in blood is not always easy to interpret and to correlate with pharmacological effect contrary to intracellular or target drug concentrations as illustrated with intralymphocyte concentrations for immunosupressants and antiretrovirals, or with CSF concentrations for antiepileptics and antidepressants. Further studies including these intracellular or target drug concentrations should be recommended to better characterise the *in vivo* impact of *ABCB1* and/or *ABCC2* SNPs and to better understand the intracellular PK-PG relationships.

In the daily clinical practice, partly because of the above mentioned reasons, there are not yet clinically validated examples of ABCB1 (or ABCC2)-genotype-directed dosing of drugs (as already proposed for some CYP, for example) but weak variations observed in drug PK with *ABCB1* and/or *ABCC2* polymorphisms, if statistically significant and confirmed in independent studies, could be integrated, as a piece of a puzzle, in predictive multivariate models (population PK models) to finally propose such ABCB1 (or ABCC2)-genotype-directed dosing guidelines.

REFERENCES

[1] Deeley RG, Westlake C, Cole SP. Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiol Rev 2006; 86: 849-99.

[2] Haufroid V, Wallemacq P, VanKerckhove V, *et al.* CYP3A5 and ABCB1 polymorphisms and tacrolimus pharmacokinetics in renal transplant candidates: guidelines from an experimental study. Am J Transplant 2006; 6: 2706-13.

[3] Kroetz DL, Pauli-Magnus C, Hodges LM, *et al.* Sequence diversity and haplotype structure in the human ABCB1 (MDR1, multidrug resistance transporter) gene. Pharmacogenetics 2003; 13: 481-94.

[4] Kimchi-Sarfaty C, Marple AH, Shinar S, *et al.* Ethnicity-related polymorphisms and haplotypes in the human ABCB1 gene. Pharmacogenomics 2007; 8: 29-39.

[5] Hoffmeyer S, Burk O, von Richter O, *et al.* Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity *in vivo*. Proc Natl Acad Sci USA 2000; 97: 3473-8.

[6] Wang D, Johnson AD, Papp AC, Kroetz DL, Sadee W. Multidrug resistance polypeptide 1 (MDR1, ABCB1) variant 3435C>T affects mRNA stability. Pharmacogenet Genom 2005; 15: 693-704.

[7] Kimchi-Sarfaty C, Oh JM, Kim IW, *et al.* A "silent" polymorphism in the MDR1 gene changes substrate specificity. Science 2007; 315: 525-8.

[8] Chowbay B, Cumaraswamy S, Cheung YB, Zhou Q, Lee EJ. Genetic polymorphisms in MDR1 and CYP3A4 genes in Asians and the influence of MDR1 haplotypes on cyclosporin disposition in heart transplant recipients. Pharmacogenetics 2003; 13: 89-95.

[9] Woodahl EL, Yang Z, Bui T, Shen DD, Ho RJ. Multidrug resistance gene G1199A polymorphism alters efflux transport activity of P-glycoprotein. J Pharmacol Exp Ther 2004; 310: 1199-207.

[10] Woodahl EL, Crouthamel MH, Bui T, Shen DD, Ho RJ. MDR1 (ABCB1) G1199A (Ser400Asn) polymorphism alters transepithelial permeability and sensitivity to anticancer agents. Cancer Chemother Pharmacol 2009; 64: 183-8.

[11] Chowbay B, Li H, David M, Cheung YB, Lee EJ. Meta-analysis of the influence of MDR1 C3435T polymorphism on digoxin pharmacokinetics and MDR1 gene expression. Br J Clin Pharmacol 2005; 60: 159-71.

[12] Johne A, Kopke K, Gerloff T, *et al.* Modulation of steady-state kinetics of digoxin by haplotypes of the P-glycoprotein MDR1 gene. Clin Pharmacol Ther 2002; 72: 584-94.

[13] Verstuyft C, Schwab M, Schaeffeler E, *et al.* Digoxin pharmacokinetics and MDR1 genetic polymorphisms. Eur J Clin Pharmacol 2003; 58: 809-12.

[14] Sakaeda T, Nakamura T, Horinouchi M, *et al.* MDR1 genotype-related pharmacokinetics of digoxin after single oral administration in healthy Japanese subjects. Pharm Res 2001; 18: 1400-4.

[15] Morita Y, Sakaeda T, Horinouchi M, *et al.* MDR1 genotype-related duodenal absorption rate of digoxin in healthy Japanese subjects. Pharm Res 2003; 20: 552-6.

[16] Larsen UL, Hyldahl OL, Guldborg NC*, et al.* Human intestinal P-glycoprotein activity estimated by the model substrate digoxin. Scand J Clin Lab Invest 2007; 67: 123-34.

[17] Comets E, Verstuyft C, Lavielle M, *et al.* Modelling the influence of MDR1 polymorphism on digoxin pharmacokinetic parameters. Eur J Clin Pharmacol 2007; 63: 437-49.

[18] Aarnoudse AJ, Dieleman JP, Visser LE, *et al.* Common ATP-binding cassette B1 variants are associated with increased digoxin serum concentration. Pharmacogenet Genom 2008; 18: 299-305.

[19] Kurzawski M, Bartnicka L, Florczak M, Gornik W, Drozdzik M. Impact of ABCB1 (MDR1) gene polymorphism and P-glycoprotein inhibitors on digoxin serum concentration in congestive heart failure patients. Pharmacol Rep 2007; 59: 107-11.

[20] Jiang ZP, Wang YR, Xu P, *et al.* Meta-analysis of the effect of MDR1 C3435T polymorphism on cyclosporine pharmacokinetics. Basic Clin Pharmacol Toxicol 2008; 103: 433-44.

[21] Anglicheau D, Thervet E, Etienne I, *et al.* CYP3A5 and MDR1 genetic polymorphisms and cyclosporine pharmacokinetics after renal transplantation. Clin Pharmacol Ther 2004; 75: 422-33.

[22] Foote CJ, Greer W, Kiberd BA, *et al.* MDR1 C3435T polymorphisms correlate with cyclosporine levels in de novo renal recipients. Transplant Proc 2006; 38: 2847-9.

[23] Foote CJ, Greer W, Kiberd B, *et al.* Polymorphisms of multidrug resistance gene (MDR1) and cyclosporine absorption in de novo renal transplant patients. Transplantation 2007; 83: 1380-4.

[24] Mai I, Stormer E, Goldammer M, *et al.* MDR1 haplotypes do not affect the steady-state pharmacokinetics of cyclosporine in renal transplant patients. J Clin Pharmacol 2003; 43: 1101-7.

[25] Min DI, Ellingrod VL. C3435T mutation in exon 26 of the human MDR1 gene and cyclosporine pharmacokinetics in healthy subjects. Ther Drug Monit 2002; 24: 400-4.

[26] Yates CR, Zhang W, Song P, *et al.* The effect of CYP3A5 and MDR1 polymorphic expression on cyclosporine oral disposition in renal transplant patients. J Clin Pharmacol 2003; 43: 555-64.

[27] von Ahsen N, Richter M, Grupp C, *et al.* No influence of the MDR-1 C3435T polymorphism or a CYP3A4 promoter polymorphism (CYP3A4-V allele) on dose-adjusted cyclosporin A trough concentrations or rejection incidence in stable renal transplant recipients. Clin Chem 2001; 47: 1048-52.

[28] Hesselink DA, van Schaik RH, van der Heiden IP, *et al.* Genetic polymorphisms of the CYP3A4, CYP3A5, and MDR-1 genes and pharmacokinetics of the calcineurin inhibitors cyclosporine and tacrolimus. Clin Pharmacol Ther 2003; 74: 245-54.

[29] Haufroid V, Mourad M, Van Kerckhove V, *et al.* The effect of CYP3A5 and MDR1 (ABCB1) polymorphisms on cyclosporine and tacrolimus dose requirements and trough blood levels in stable renal transplant patients. Pharmacogenetics 2004; 14: 147-54.

[30] Hu YF, Qiu W, Liu ZQ, *et al.* Effects of genetic polymorphisms of CYP3A4, CYP3A5 and MDR1 on cyclosporine pharmacokinetics after renal transplantation. Clin Exp Pharmacol Physiol 2006; 33: 1093-8.

[31] Crettol S, Venetz JP, Fontana M, *et al.* Influence of ABCB1 genetic polymorphisms on cyclosporine intracellular concentration in transplant recipients. Pharmacogenet Genom 2008; 18: 307-15.

[32] Hauser IA, Schaeffeler E, Gauer S, *et al.* ABCB1 genotype of the donor but not of the recipient is a major risk factor for cyclosporine-related nephrotoxicity after renal transplantation. J Am Soc Nephrol 2005; 16: 1501-11.

[33] Kuehl P, Zhang J, Lin Y, *et al.* Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. Nat Genet 2001; 27: 383-91.

[34] Hesselink DA, van Gelder T, van Schaik RH. The pharmacogenetics of calcineurin inhibitors: one step closer toward individualized immunosuppression? Pharmacogenomics 2005; 6: 323-37.

[35] Tsuchiya N, Satoh S, Tada H, *et al.* Influence of CYP3A5 and MDR1 (ABCB1) polymorphisms on the pharmacokinetics of tacrolimus in renal transplant recipients. Transplantation 2004; 78: 1182-7.

[36] Zhang X, Liu ZH, Zheng JM, *et al.* Influence of CYP3A5 and MDR1 polymorphisms on tacrolimus concentration in the early stage after renal transplantation. Clin Transplant 2005; 19: 638-43.

[37] Mourad M, Wallemacq P, De Meyer M, *et al.* The influence of genetic polymorphisms of cytochrome P450 3A5 and ABCB1 on starting dose- and weight-standardized tacrolimus trough concentrations after kidney transplantation in relation to renal function. Clin Chem Lab Med 2006; 44: 1192-8.

[38] Choi JH, Lee YJ, Jang SB, *et al.* Influence of the CYP3A5 and MDR1 genetic polymorphisms on the pharmacokinetics of tacrolimus in healthy Korean subjects. Br J Clin Pharmacol 2007; 64: 185-91.

[39] Kuypers DR, de Jonge H, Naesens M, *et al.* CYP3A5 and CYP3A4 but not MDR1 single-nucleotide polymorphisms determine long-term tacrolimus disposition and drug-related nephrotoxicity in renal recipients. Clin Pharmacol Ther 2007; 82: 711-25.

[40] Li D, Zhu JY, Gao J, *et al.* Polymorphisms of tumor necrosis factor-alpha, interleukin-10, cytochrome P450 3A5 and ABCB1 in Chinese liver transplant patients treated with immunosuppressant tacrolimus. Clin Chim Acta 2007; 383: 133-9.

[41] Op den Buijsch RA, Christiaans MH, Stolk LM, *et al.* Tacrolimus pharmacokinetics and pharmacogenetics: influence of adenosine triphosphate-binding cassette B1 (ABCB1) and cytochrome (CYP) 3A polymorphisms. Fundam Clin Pharmacol 2007; 21: 427-35.

[42] Quteineh L, Verstuyft C, Furlan V, *et al.* Influence of CYP3A5 genetic polymorphism on tacrolimus daily dose requirements and acute rejection in renal graft recipients. Basic Clin Pharmacol Toxicol 2008; 103: 546-52.

[43] Provenzani A, Notarbartolo M, Labbozzetta M, *et al.* The effect of CYP3A5 and ABCB1 single nucleotide polymorphisms on tacrolimus dose requirements in Caucasian liver transplant patients. Ann Transplant 2009; 14: 23-31.

[44] Mai I, Perloff ES, Bauer S, *et al.* MDR1 haplotypes derived from exons 21 and 26 do not affect the steady-state pharmacokinetics of tacrolimus in renal transplant patients. Br J Clin Pharmacol 2004; 58: 548-53.

[45] Zheng H, Webber S, Zeevi A, *et al.* Tacrolimus dosing in pediatric heart transplant patients is related to CYP3A5 and MDR1 gene polymorphisms. Am J Transplant 2003; 3: 477-83.

[46] Zheng H, Schuetz E, Zeevi A, *et al.* Sequential analysis of tacrolimus dosing in adult lung transplant patients with ABCB1 haplotypes. J Clin Pharmacol 2005; 45: 404-10.

[47] Wei-lin W, Jing J, Shu-sen Z, *et al.* Tacrolimus dose requirement in relation to donor and recipient ABCB1 and CYP3A5 gene polymorphisms in Chinese liver transplant patients. Liver Transpl 2006; 12: 775-80.

[48] Macphee IA, Fredericks S, Tai T, *et al.* Tacrolimus pharmacogenetics: polymorphisms associated with expression of cytochrome p4503A5 and P-glycoprotein correlate with dose requirement. Transplantation 2002; 74: 1486-9.

[49] Anglicheau D, Verstuyft C, Laurent-Puig P, *et al.* Association of the multidrug resistance-1 gene single-nucleotide polymorphisms with the tacrolimus dose requirements in renal transplant recipients. J Am Soc Nephrol 2003; 14: 1889-96.

[50] Cheung CY, Op den Buijsch RA, Wong KM, *et al.* Influence of different allelic variants of the CYP3A and ABCB1 genes on the tacrolimus pharmacokinetic profile of Chinese renal transplant recipients. Pharmacogenomics 2006; 7: 563-74.

[51] Akbas SH, Bilgen T, Keser I, *et al.* The effect of MDR1 (ABCB1) polymorphism on the pharmacokinetic of tacrolimus in Turkish renal transplant recipients. Transplant Proc 2006; 38: 1290-2.

[52] Roy JN, Barama A, Poirier C, Vinet B, Roger M. Cyp3A4, Cyp3A5, and MDR-1 genetic influences on tacrolimus pharmacokinetics in renal transplant recipients. Pharmacogenet Genom 2006; 16: 659-65.

[53] Li D, Gui R, Li J, Huang Z, Nie X. Tacrolimus dosing in Chinese renal transplant patients is related to MDR1 gene C3435T polymorphisms. Transplant Proc 2006; 38: 2850-2.

[54] Tada H, Tsuchiya N, Satoh S, *et al.* Impact of CYP3A5 and MDR1(ABCB1) C3435T polymorphisms on the pharmacokinetics of tacrolimus in renal transplant recipients. Transplant Proc 2005; 37: 1730-2.

[55] Fredericks S, Moreton M, Reboux S, *et al.* Multidrug resistance gene-1 (MDR-1) haplotypes have a minor influence on tacrolimus dose requirements. Transplantation 2006; 82: 705-8.

[56] Wang J, Zeevi A, McCurry K, *et al.* Impact of ABCB1 (MDR1) haplotypes on tacrolimus dosing in adult lung transplant patients who are CYP3A5 *3/*3 non-expressors. Transpl Immunol 2006; 15: 235-40.

[57] Capron A, Lerut J, Verbaandert C, *et al.* Validation of a liquid chromatography-mass spectrometric assay for tacrolimus in liver biopsies after hepatic transplantation: correlation with histopathologic staging of rejection. Ther Drug Monit 2007; 29: 340-8.

[58] Elens L, Capron A, Kerckhove VV, *et al.* 1199G>A and 2677G>T/A polymorphisms of ABCB1 independently affect tacrolimus concentration in hepatic tissue after liver transplantation. Pharmacogenet Genom 2007; 17: 873-83.

[59] Anglicheau D, Le Corre D, Lechaton S, *et al.* Consequences of genetic polymorphisms for sirolimus requirements after renal transplant in patients on primary sirolimus therapy. Am J Transplant 2005; 5: 595-603.

[60] Mourad M, Mourad G, Wallemacq P, *et al.* Sirolimus and tacrolimus trough concentrations and dose requirements after kidney transplantation in relation to CYP3A5 and MDR1 polymorphisms and steroids. Transplantation 2005; 80: 977-84.

[61] Renders L, Frisman M, Ufer M, *et al.* CYP3A5 genotype markedly influences the pharmacokinetics of tacrolimus and sirolimus in kidney transplant recipients. Clin Pharmacol Ther 2007; 81: 228-34.

[62] Miao LY, Huang CR, Hou JQ, Qian MY. Association study of ABCB1 and CYP3A5 gene polymorphisms with sirolimus trough concentration and dose requirements in Chinese renal transplant recipients. Biopharm Drug Dispos 2008; 29: 1-5.

[63] Goh BC, Lee SC, Wang LZ, *et al.* Explaining interindividual variability of docetaxel pharmacokinetics and pharmacodynamics in Asians through phenotyping and genotyping strategies. J Clin Oncol 2002; 20: 3683-90.

[64] Tran A, Jullien V, Alexandre J, *et al.* Pharmacokinetics and toxicity of docetaxel: role of CYP3A, MDR1, and GST polymorphisms. Clin Pharmacol Ther 2006; 79: 570-80.

[65] Bosch TM, Huitema AD, Doodeman VD, *et al.* Pharmacogenetic screening of CYP3A and ABCB1 in relation to population pharmacokinetics of docetaxel. Clin Cancer Res 2006; 12: 5786-93.

[66] Nakajima M, Fujiki Y, Kyo S, *et al.* Pharmacokinetics of paclitaxel in ovarian cancer patients and genetic polymorphisms of CYP2C8, CYP3A4, and MDR1. J Clin Pharmacol 2005; 45: 674-82.

[67] Henningsson A, Marsh S, Loos WJ, *et al.* Association of CYP2C8, CYP3A4, CYP3A5, and ABCB1 polymorphisms with the pharmacokinetics of paclitaxel. Clin Cancer Res 2005; 11: 8097-104.

[68] Jiko M, Yano I, Sato E, *et al.* Pharmacokinetics and pharmacodynamics of paclitaxel with carboplatin or gemcitabine, and effects of CYP3A5 and MDR1 polymorphisms in patients with urogenital cancers. Int J Clin Oncol 2007; 12: 284-90.

[69] Plasschaert SL, Groninger E, Boezen M, *et al.* Influence of functional polymorphisms of the MDR1 gene on vincristine pharmacokinetics in childhood acute lymphoblastic leukemia. Clin Pharmacol Ther 2004; 76: 220-9.

[70] Sai K, Kaniwa N, Itoda M, *et al.* Haplotype analysis of ABCB1/MDR1 blocks in a Japanese population reveals genotype-dependent renal clearance of irinotecan. Pharmacogenetics 2003; 13: 741-57.

[71] Han JY, Lim HS, Yoo YK, *et al.* Associations of ABCB1, ABCC2, and ABCG2 polymorphisms with irinotecan-pharmacokinetics and clinical outcome in patients with advanced non-small cell lung cancer. Cancer 2007; 110: 138-47.

[72] Gardner ER, Burger H, van Schaik RH, *et al.* Association of enzyme and transporter genotypes with the pharmacokinetics of imatinib. Clin Pharmacol Ther 2006; 80: 192-201.

[73] Gurney H, Wong M, Balleine RL, *et al.* Imatinib disposition and ABCB1 (MDR1, P-glycoprotein) genotype. Clin Pharmacol Ther 2007; 82: 33-40.

[74] Dulucq S, Bouchet S, Turcq B, *et al.* Multidrug resistance gene (MDR1) polymorphisms are associated with major molecular responses to standard-dose imatinib in chronic myeloid leukemia. Blood 2008; 112: 2024-7.

[75] Lal S, Wong ZW, Sandanaraj E, *et al.* Influence of ABCB1 and ABCG2 polymorphisms on doxorubicin disposition in Asian breast cancer patients. Cancer Sci 2008; 99: 816-23.

[76] Winzer R, Langmann P, Zilly M, *et al.* No influence of the P-glycoprotein genotype (MDR1 C3435T) on plasma levels of lopinavir and efavirenz during antiretroviral treatment. Eur J Med Res 2003; 8: 531-4.

[77] Ma Q, Brazeau D, Zingman BS, *et al.* Multidrug resistance 1 polymorphisms and trough concentrations of atazanavir and lopinavir in patients with HIV. Pharmacogenomics 2007; 8: 227-35.

[78] Estrela RC, Ribeiro FS, Barroso PF, *et al.* ABCB1 polymorphisms and the concentrations of lopinavir and ritonavir in blood, semen and saliva of HIV-infected men under antiretroviral therapy. Pharmacogenomics 2009; 10: 311-8.

[79] Frohlich M, Burhenne J, Martin-Facklam M, *et al.* Oral contraception does not alter single dose saquinavir pharmacokinetics in women. Br J Clin Pharmacol 2004; 57: 244-52.

[80] Mouly SJ, Matheny C, Paine MF, *et al.* Variation in oral clearance of saquinavir is predicted by CYP3A5*1 genotype but not by enterocyte content of cytochrome P450 3A5. Clin Pharmacol Ther 2005; 78: 605-18.

[81] la Porte CJ, Li Y, Beique L, *et al.* The effect of ABCB1 polymorphism on the pharmacokinetics of saquinavir alone and in combination with ritonavir. Clin Pharmacol Ther 2007; 82: 389-95.

[82] Verstuyft C, Marcellin F, Morand-Joubert L, *et al.* Absence of association between MDR1 genetic polymorphisms, indinavir pharmacokinetics and response to highly active antiretroviral therapy. AIDS 2005; 19: 2127-31.

[83] Solas C, Simon N, Drogoul MP, *et al.* Minimal effect of MDR1 and CYP3A5 genetic polymorphisms on the pharmacokinetics of

indinavir in HIV-infected patients. Br J Clin Pharmacol 2007; 64: 353-62.

[84] Curras V, Hocht C, Mangano A, *et al.* Pharmacokinetic study of the variability of indinavir drug levels when boosted with ritonavir in HIV-infected children. Pharmacology 2009; 83: 59-66.

[85] Rodriguez NS, Barreiro P, Rendon A, *et al.* Plasma levels of atazanavir and the risk of hyperbilirubinemia are predicted by the 3435C-->T polymorphism at the multidrug resistance gene 1. Clin Infect Dis 2006; 42: 291-5.

[86] Rodriguez-Novoa S, Martin-Carbonero L, Barreiro P, *et al.* Genetic factors influencing atazanavir plasma concentrations and the risk of severe hyperbilirubinemia. AIDS 2007; 21: 41-6.

[87] Hirt D, Mentre F, Tran A, *et al.* Effect of CYP2C19 polymorphism on nelfinavir to M8 biotransformation in HIV patients. Br J Clin Pharmacol 2008; 65: 548-57.

[88] Saitoh A, Singh KK, Powell CA, *et al.* An MDR1-3435 variant is associated with higher plasma nelfinavir levels and more rapid virologic response in HIV-1 infected children. AIDS 2005; 19: 371-80.

[89] Colombo S, Soranzo N, Rotger M, *et al.* Influence of ABCB1, ABCC1, ABCC2, and ABCG2 haplotypes on the cellular exposure of nelfinavir *in vivo*. Pharmacogenet Genomics 2005; 15: 599-608.

[90] Saitoh A, Sarles E, Capparelli E, *et al.* CYP2B6 genetic variants are associated with nevirapine pharmacokinetics and clinical response in HIV-1-infected children. AIDS 2007; 21: 2191-9.

[91] Mahungu T, Smith C, Turner F, *et al.* Cytochrome P450 2B6 516G-->T is associated with plasma concentrations of nevirapine at both 200 mg twice daily and 400 mg once daily in an ethnically diverse population. HIV Med 2009; 10: 310-7.

[92] Stormer E, von Moltke LL, Perloff MD, Greenblatt DJ. Differential modulation of P-glycoprotein expression and activity by non-nucleoside HIV-1 reverse transcriptase inhibitors in cell culture. Pharm Res 2002; 19: 1038-45.

[93] Zhang Y, Jiang XH, Hu YQ, *et al.* MDR1 genotypes do not influence the absorption of a single oral dose of 600 mg valacyclovir in healthy Chinese Han ethnic males. Br J Clin Pharmacol 2008; 66: 247-54.

[94] Sansone-Parsons A, Krishna G, Simon J, *et al.* Effects of age, gender, and race/ethnicity on the pharmacokinetics of posaconazole in healthy volunteers. Antimicrob Agents Chemother 2007; 51: 495-502.

[95] Weiss J, Ten Hoevel MM, Burhenne J, *et al.* CYP2C19 genotype is a major factor contributing to the highly variable pharmacokinetics of voriconazole. J Clin Pharmacol 2009; 49: 196-204.

[96] Putnam WS, Woo JM, Huang Y, Benet LZ. Effect of the MDR1 C3435T variant and P-glycoprotein induction on dicloxacillin pharmacokinetics. J Clin Pharmacol 2005; 45: 411-21.

[97] Yin OQ, Tomlinson B, Chow MS. Effect of multidrug resistance gene-1 (ABCB1) polymorphisms on the single-dose pharmacokinetics of cloxacillin in healthy adult Chinese men. Clin Ther 2009; 31: 999-1006.

[98] Kerb R, Aynacioglu AS, Brockmoller J, *et al.* The predictive value of MDR1, CYP2C9, and CYP2C19 polymorphisms for phenytoin plasma levels. Pharmacogenom J 2001; 1: 204-10.

[99] Ebid AH, Ahmed MM, Mohammed SA. Therapeutic drug monitoring and clinical outcomes in epileptic Egyptian patients: a gene polymorphism perspective study. Ther Drug Monit 2007; 29: 305-12.

[100] Seo T, Ishitsu T, Ueda N, *et al.* ABCB1 polymorphisms influence the response to antiepileptic drugs in Japanese epilepsy patients. Pharmacogenomics 2006; 7: 551-61.

[101] Basic S, Hajnsek S, Bozina N, *et al.* The influence of C3435T polymorphism of ABCB1 gene on penetration of phenobarbital across the blood-brain barrier in patients with generalized epilepsy. Seizure 2008; 17: 524-30.

[102] Yasui-Furukori N, Mihara K, Takahata T, *et al.* Effects of various factors on steady-state plasma concentrations of risperidone and 9-hydroxyrisperidone: lack of impact of MDR-1 genotypes. Br J Clin Pharmacol 2004; 57: 569-75.

[103] Xing Q, Gao R, Li H, *et al.* Polymorphisms of the ABCB1 gene are associated with the therapeutic response to risperidone in Chinese schizophrenia patients. Pharmacogenomics 2006; 7: 987-93.

[104] Gunes A, Spina E, Dahl ML, Scordo MG. ABCB1 Polymorphisms Influence Steady-State Plasma Levels of 9-Hydroxyrisperidone and Risperidone Active Moiety. Ther Drug Monit 2008; 30: 628-33.

[105] Rahi M, Heikkinen T, Hartter S, *et al.* Placental transfer of quetiapine in relation to P-glycoprotein activity. J Psychopharmacol 2007; 21: 751-6.

[106] Jaquenoud SE, Knezevic B, Morena GP, *et al.* ABCB1 and cytochrome P450 polymorphisms: clinical pharmacogenetics of clozapine. J Clin Psychopharmacol 2009; 29: 319-26.

[107] Fukui N, Suzuki Y, Sawamura K, *et al.* Dose-dependent effects of the 3435 C>T genotype of ABCB1 gene on the steady-state plasma concentration of fluvoxamine in psychiatric patients. Ther Drug Monit 2007; 29: 185-9.

[108] Gex-Fabry M, Eap CB, Oneda B, *et al.* CYP2D6 and ABCB1 genetic variability: influence on paroxetine plasma level and therapeutic response. Ther Drug Monit 2008; 30: 474-82.

[109] Nikisch G, Eap CB, Baumann P. Citalopram enantiomers in plasma and cerebrospinal fluid of ABCB1 genotyped depressive patients and clinical response: a pilot study. Pharmacol Res 2008; 58: 344-7.

[110] Seeringer A, Kirchheiner J. Pharmacogenetics-guided dose modifications of antidepressants. Clin Lab Med 2008; 28: 619-26.

[111] Pauli-Magnus C, Feiner J, Brett C, Lin E, Kroetz DL. No effect of MDR1 C3435T variant on loperamide disposition and central nervous system effects. Clin Pharmacol Ther 2003; 74: 487-98.

[112] Skarke C, Jarrar M, Schmidt H, *et al.* Effects of ABCB1 (multidrug resistance transporter) gene mutations on disposition and central nervous effects of loperamide in healthy volunteers. Pharmacogenetics 2003; 13: 651-60.

[113] Crettol S, Deglon JJ, Besson J, *et al.* ABCB1 and cytochrome P450 genotypes and phenotypes: influence on methadone plasma levels and response to treatment. Clin Pharmacol Ther 2006; 80: 668-81.

[114] Levran O, O'Hara K, Peles E, *et al.* ABCB1 (MDR1) genetic variants are associated with methadone doses required for effective treatment of heroin dependence. Hum Mol Genet 2008; 17: 2219-27.

[115] Coller JK, Barratt DT, Dahlen K, Loennechen MH, Somogyi AA. ABCB1 genetic variability and methadone dosage requirements in opioid-dependent individuals. Clin Pharmacol Ther 2006; 80: 682-90.

[116] Siegmund W, Ludwig K, Giessmann T, *et al.* The effects of the human MDR1 genotype on the expression of duodenal P-glycoprotein and disposition of the probe drug talinolol. Clin Pharmacol Ther 2002; 72: 572-83.

[117] Zhang WX, Chen GL, Zhang W, *et al.* MDR1 genotype do not influence the absorption of a single oral dose of 100 mg talinolol in healthy Chinese males. Clin Chim Acta 2005; 359: 46-52.

[118] Yasar U, Babaoglu MO, Bozkurt A. Disposition of a CYP2C9 phenotyping agent, losartan, is not influenced by the common 3435C > T variation of the drug transporter gene ABCB1 (MDR1). Basic Clin Pharmacol Toxicol 2008; 103: 176-9.

[119] Guo X, Chen XP, Cheng ZN, *et al.* No effect of MDR1 C3435T polymorphism on oral pharmacokinetics of telmisartan in 19 healthy Chinese male subjects. Clin Chem Lab Med 2009; 47: 38-43.

[120] Brunner M, Langer O, Sunder-Plassmann R, *et al.* Influence of functional haplotypes in the drug transporter gene ABCB1 on central nervous system drug distribution in humans. Clin Pharmacol Ther 2005; 78: 182-90.

[121] Kim KA, Park PW, Park JY. Effect of ABCB1 (MDR1) haplotypes derived from G2677T/C3435T on the pharmacokinetics of amlodipine in healthy subjects. Br J Clin Pharmacol 2007; 63: 53-8.

[122] Keskitalo JE, Kurkinen KJ, Neuvonen M, *et al.* No significant effect of ABCB1 haplotypes on the pharmacokinetics of fluvastatin, pravastatin, lovastatin, and rosuvastatin. Br J Clin Pharmacol 2009; 68: 207-13.

[123] Niemi M, Schaeffeler E, Lang T, *et al.* High plasma pravastatin concentrations are associated with single nucleotide polymorphisms and haplotypes of organic anion transporting polypeptide-C (OATP-C, SLCO1B1). Pharmacogenetics 2004; 14: 429-40.

[124] Hedman M, Antikainen M, Holmberg C, *et al.* Pharmacokinetics and response to pravastatin in paediatric patients with familial hypercholesterolaemia and in paediatric cardiac transplant recipients in relation to polymorphisms of the SLCO1B1 and ABCB1 genes. Br J Clin Pharmacol 2006; 61: 706-15.

[125] Keskitalo JE, Kurkinen KJ, Neuvoneni PJ, Niemi M. ABCB1 haplotypes differentially affect the pharmacokinetics of the acid

and lactone forms of simvastatin and atorvastatin. Clin Pharmacol Ther 2008; 84: 457-61.

[126] Niemi M. Role of OATP transporters in the disposition of drugs. Pharmacogenomics 2007; 8: 787-802.

[127] Taubert D, von Beckerath N, Grimberg G, *et al.* Impact of P-glycoprotein on clopidogrel absorption. Clin Pharmacol Ther 2006; 80: 486-501.

[128] Drescher S, Schaeffeler E, Hitzl M, *et al.* MDR1 gene polymorphisms and disposition of the P-glycoprotein substrate fexofenadine. Br J Clin Pharmacol 2002; 53: 526-34.

[129] Yi SY, Hong KS, Lim HS, *et al.* A variant 2677A allele of the MDR1 gene affects fexofenadine disposition. Clin Pharmacol Ther 2004; 76: 418-27.

[130] Miura M, Satoh S, Tada H, *et al.* Influence of ABCB1 C3435T polymorphism on the pharmacokinetics of lansoprazole and gastroesophageal symptoms in Japanese renal transplant recipients classified as CYP2C19 extensive metabolizers and treated with tacrolimus. Int J Clin Pharmacol Ther 2006; 44: 605-13.

[131] Ufer M, Dilger K, Leschhorn L, *et al.* Influence of CYP3A4, CYP3A5, and ABCB1 genotype and expression on budesonide pharmacokinetics: a possible role of intestinal CYP3A4 expression. Clin Pharmacol Ther 2008; 84: 43-6.

[132] Miura M, Satoh S, Inoue K, *et al.* Influence of CYP3A5, ABCB1 and NR1I2 polymorphisms on prednisolone pharmacokinetics in renal transplant recipients. Steroids 2008; 73: 1052-9.

[133] Suzuki H, Sugiyama Y. Single nucleotide polymorphisms in multidrug resistance associated protein 2 (MRP2/ABCC2): its impact on drug disposition. Adv Drug Deliv Rev 2002; 54: 1311-31.

[134] Oselin K, Mrozikiewicz PM, Pahkla R, Roots I. Quantitative determination of the human MRP1 and MRP2 mRNA expression in FACS-sorted peripheral blood CD4+, CD8+, CD19+, and CD56+ cells. Eur J Haematol 2003; 71: 119-23.

[135] Haenisch S, Zimmermann U, Dazert E, *et al.* Influence of polymorphisms of ABCB1 and ABCC2 on mRNA and protein expression in normal and cancerous kidney cortex. Pharmacogenom J 2007; 7: 56-65.

[136] Naesens M, Kuypers DR, Verbeke K, Vanrenterghem Y. Multidrug resistance protein 2 genetic polymorphisms influence mycophenolic acid exposure in renal allograft recipients. Transplantation 2006; 82: 1074-84.

[137] Miura M, Satoh S, Inoue K, *et al.* Influence of SLCO1B1, 1B3, 2B1 and ABCC2 genetic polymorphisms on mycophenolic acid pharmacokinetics in Japanese renal transplant recipients. Eur J Clin Pharmacol 2007; 63: 1161-9.

[138] Baldelli S, Merlini S, Perico N, *et al.* C-440T/T-331C polymorphisms in the UGT1A9 gene affect the pharmacokinetics of mycophenolic acid in kidney transplantation. Pharmacogenomics 2007; 8: 1127-41.

[139] Levesque E, Benoit-Biancamano MO, Delage R, Couture F, Guillemette C. Pharmacokinetics of mycophenolate mofetil and its glucuronide metabolites in healthy volunteers. Pharmacogenomics 2008; 9: 869-79.

[140] Zhang WX, Chen B, Jin Z, *et al.* Influence of uridine diphosphate (UDP)-glucuronosyltransferases and ABCC2 genetic polymorphisms on the pharmacokinetics of mycophenolic acid and its metabolites in Chinese renal transplant recipients. Xenobiotica 2008; 38: 1422-36.

[141] Rau T, Erney B, Gores R, *et al.* High-dose methotrexate in pediatric acute lymphoblastic leukemia: impact of ABCC2 polymorphisms on plasma concentrations. Clin Pharmacol Ther 2006; 80: 468-76.

[142] Innocenti F, Kroetz DL, Schuetz E, *et al.* Comprehensive pharmacogenetic analysis of irinotecan neutropenia and pharmacokinetics. J Clin Oncol 2009; 27: 2604-14.

[143] Fujita K, Nagashima F, Yamamoto W, *et al.* Association of ATP-binding cassette, sub-family C, number 2 (ABCC2) genotype with pharmacokinetics of irinotecan in Japanese patients with metastatic colorectal cancer treated with irinotecan plus infusional 5-fluorouracil/leucovorin (FOLFIRI). Biol Pharm Bull 2008; 31: 2137-42.

[144] de Jong FA, Scott-Horton TJ, Kroetz DL, *et al.* Irinotecan-induced diarrhea: functional significance of the polymorphic ABCC2 transporter protein. Clin Pharmacol Ther 2007; 81: 42-9.

[145] Zamboni WC, Ramanathan RK, McLeod HL, *et al.* Disposition of 9-nitrocamptothecin and its 9-aminocamptothecin metabolite in relation to ABC transporter genotypes. Invest New Drugs 2006; 24: 393-401.

[146] Kiser JJ, Aquilante CL, Anderson PL, *et al.* Clinical and genetic determinants of intracellular tenofovir diphosphate concentrations in HIV-infected patients. J Acquir Immune Defic Syndr 2008; 47: 298-303.

[147] Elens L, Yombi JC, Lison D, *et al.* Association between ABCC2 polymorphism and lopinavir accumulation in peripheral blood mononuclear cells of HIV-infected patients. Pharmacogenomics 2009; 10: 1589-97.

[148] Ho RH, Choi L, Lee W, *et al.* Effect of drug transporter genotypes on pravastatin disposition in European- and African-American participants. Pharmacogenet Genomics 2007; 17: 647-56.

[149] Bernsdorf A, Giessmann T, Modess C, *et al.* Simvastatin does not influence the intestinal P-glycoprotein and MPR2, and the disposition of talinolol after chronic medication in healthy subjects genotyped for the ABCB1, ABCC2 and SLCO1B1 polymorphisms. Br J Clin Pharmacol 2006; 61: 440-50.

[150] Niemi M, Arnold KA, Backman JT, *et al.* Association of genetic polymorphism in ABCC2 with hepatic multidrug resistance-associated protein 2 expression and pravastatin pharmacokinetics. Pharmacogenet Genomics 2006; 16: 801-8.

[151] Haenisch S, May K, Wegner D, *et al.* Influence of genetic polymorphisms on intestinal expression and rifampicin-type induction of ABCC2 and on bioavailability of talinolol. Pharmacogenet Genomics 2008; 18: 357-65.

[152] Miura M, Satoh S, Inoue K, *et al.* Telmisartan pharmacokinetics in Japanese renal transplant recipients. Clin Chem Acta 2009; 399: 83-87.

[153] Fromm M. P-glycoprotein: a defense mechanism limiting oral bioavailability and CNS accumulation of drugs. Int J Clin Pharm Ther 2000; 38: 69-74.

Received: February 05, 2010 Revised: August 13, 2010 Accepted: August 13, 2010

ATP-Binding Cassette Transporters A1 and G1, HDL Metabolism, Cholesterol Efflux, and Inflammation: Important Targets for the Treatment of Atherosclerosis

Dan Ye*, Bart Lammers, Ying Zhao, Illiana Meurs, Theo Van Berkel, Miranda Van Eck

Division of Biopharmaceutics, Leiden University, Leiden, The Netherlands

Abstract: Atherosclerosis has been characterized as a chronic inflammatory response to cholesterol deposition in arteries. Plasma high density lipoprotein (HDL) levels bear a strong independent inverse relationship with atherosclerotic cardiovascular disease. One central antiatherogenic role of HDL is believed to be its ability to remove excessive peripheral cholesterol back to the liver for subsequent catabolism and excretion, a physiologic process termed reverse cholesterol transport (RCT). Cholesterol efflux from macrophage foam cells, the initial step of RCT is the most relevant step with respect to atherosclerosis. The ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 play crucial roles in the efflux of cellular cholesterol to HDL and its apolipoproteins. Moreover, ABCA1 and ABCG1 affect cellular inflammatory cytokine secretion by modulating cholesterol content in the plasma membrane and within intracellular compartments. In humans, ABCA1 mutations can cause a severe HDL-deficiency syndrome characterized by cholesterol deposition in tissue macrophages and prevalent atherosclerosis. Disrupting Abca1 or Abcg1 in mice promotes accumulation of excessive cholesterol in macrophages, and physiological manipulation of ABCA1 expression affects atherogenesis.

Here we review recent advances in the role of ABCA1 and ABCG1 in HDL metabolism, macrophage cholesterol efflux, inflammation, and atherogenesis. Next, we summarize the structure, expression, and regulation of ABCA1 and ABCG1. Finally, we give an update on the progress and pitfalls of therapeutic approaches that target ABCA1 and ABCG1 to stimulate the flux of lipids through the RCT pathway.

Keywords: Atherosclerosis, macrophages, cholesterol, inflammation, ABCA1, ABCG1.

INTRODUCTION

Plasma levels of high density lipoprotein (HDL) are inversely proportional to the risk of atherosclerotic cardiovascular disease (CVD) in humans [1]. The key mechanism underlying the atheroprotective properties of HDL is thought to be its role in reverse cholesterol transport (RCT), a physiologic process by which excess peripheral cholesterol is transported by HDL to the liver for excretion in the bile and feces [2]. In addition, HDL can prevent oxidation of low density lipoprotein (LDL) [3], act as an anticoagulant [4] and have anti-inflammatory properties [5], all of which may contribute to the antiatherogenic effects of HDL. This presumption leads to an assertion that raising HDL cholesterol (HDL-C) levels alone would improve RCT and protect against the development of atherosclerosis. However, high HDL-C concentration appears not to be always synonymous with an efficient RCT. Therefore, improving the HDL functionality may be more attractive to potentially stimulate RCT.

The initial step of RCT is thought to be dependent upon extracellular lipid acceptors, including HDL and/or lipid-poor apolipoproteins. Studies have indicated that the ATP-binding cassette (ABC) transporter ABCA1 is required for the maintenance of normal plasma HDL-C levels, and is important for the first step of RCT, i.e., transfer of cholesterol to lipid-poor HDL apolipoproteins (e.g., apoA-I) [6]. ABCG1 is also of interest, since it participates in an intermediate RCT step, i.e. transfer of cholesterol to mature HDL particles (e.g., HDL2 and HDL3) [7]. Moreover, ABCA1 and ABCG1 export lipids to either cell surface-bound or internalized apolipoproteins [8, 9]. Minor changes in cholesterol content in the plasma membrane or within intracellular vesicular compartments can significantly impact cell function via influencing cellular signaling events [10, 11]. Thus, the traditional role of ABCA1 and ABCG1 in lipid transport is mechanistically linked to their potential role in suppressing inflammation.

Here we review recent advances in the role of ABCA1 and ABCG1 in HDL metabolism, macrophage cholesterol efflux, inflammation, and atherogenesis. Next, we summarize the structure, expression, and regulation of ABCA1 and ABCG1. Finally, we attempt to integrate this information and discuss the progress and pitfalls of therapeutic approaches that target ABCA1 and ABCG1 to stimulate the flux of lipids through the RCT pathway.

1. ABCA1 AND ABCG1 IN HDL METABOLISM AND CONSEQUENCES FOR ATHEROSCLEROSIS

1.1. ABCA1, HDL, and Atherosclerosis

ABCA1, first known as ABC1, was cloned in 1994 by Luciani and colleagues [12] as a homolog of yeast ced-7, and is a member of the large superfamily of ABC transporters that use ATP as an energy source to transport lipids and other molecules across membranes [13]. Understanding of

*Address correspondence to this author at the LACDR, Division of Biopharmaceutics, Einsteinweg 55, 2333 CC Leiden, The Netherlands; Tel: +31-71-5276238; Fax: +31-71-5276032; E-mail: y.dan@chem.leidenuniv.nl

1389-4501/11 $58.00+.00 © 2011 Bentham Science Publishers Ltd.

the role of ABCA1 in HDL biogenesis came with the discovery that mutations in the ABCA1 gene result in the extreme HDL deficiency syndrome Tangier disease (TD). In 1999, the mutation in TD was mapped to chromosome 9q31 in the ABCA1 gene [14-17]. Since then, an enormous amount of research has been set off to investigate the mechanism of action, regulation, and suitability of ABCA1 as a target to increase HDL formation therapeutically for the treatment and prevention of atherosclerosis.

The defective cholesterol and phospholipid efflux to apoA-I from fibroblasts derived from TD patients suggested that the removal of cellular lipids to apoA-I is a major predictor of plasma HDL-C levels [18, 19]. Although the structure and synthetic rates of apoA-I are normal in TD, the hypercatabolism of circulating apoA-I may result in the severe HDL deficiency syndrome in this disease [20]. As a result, homozygous TD is associated with extremely low levels of apoA-I (<1% normal) and HDL-C (<5% normal). In accord, several recent genome-wide association studies have identified common variants in ABCA1 as a significant source of variation in plasma HDL-C levels across multiple ethnic groups [21-23], establishing ABCA1 as a major gene influencing HDL levels in humans. However, the impact of ABCA1 on atherosclerosis remains controversial. An early systematic survey of TD patients indicated homozygotes over the age of 30 displaying a 6-fold higher incidence of CVD than normolipidemic control subjects [24]. Of note, the increased risk of atherosclerosis in TD patients does not appear to be as dramatic as one would expect in individuals with an almost complete absence of HDL. One possible explanation is that TD homozygotes in addition to having low HDL-C also have reduced levels of VLDL and LDL cholesterol (VLDL-C and LDL-C), and thus the atherogenic stimulus may be diminished. Comparison of the phenotypes of TD homozygotes with heterozygotes who have decreased HDL-C (≈40% normal) but normal VLDL-C or LDL-C levels confirmed that ABCA1 is a rate-limiting factor in HDL biosynthesis, while one functional ABCA1 allele is sufficient to maintain normal LDL levels. Moreover, heterozygotes do not display the excessive accumulation of lipid-laden tissue macrophages as observed in homozygotes [25], implying that one functional ABCA1 allele is sufficient to prevent excessive cholesterol accumulation in macrophages. Interestingly, heterozygotes also showed significant increases in CVD incidence and carotid artery intima media thickness as compared to healthy controls [26]. One study of the Copenhagen City Heart cohort revealed that genetic variations in ABCA1 affect CVD in the general population [27]. However, a follow up study of the same cohort concluded that low HDL caused by loss-of-function mutations in ABCA1 does not contribute to the risk of CVD [28]. It was also reported that both common and rare ABCA1 variations are associated with increased CVD despite normal HDL levels [27, 29, 30]. Collectively, these studies support the idea that impaired ABCA1 function increases atherosclerosis in humans, although not in all cases by mechanisms that reflect reduced plasma HDL-C levels.

Studies in mice where the Abca1 locus is deleted or human ABCA1 is overexpressed generally support the hypothesis that ABCA1 by maintaining circulating HDL-C levels and cellular cholesterol efflux significantly prevents atherosclerosis. Total-body Abca1-deficient ($Abca1^{-/-}$) mice exhibit low plasma HDL-C levels and cholesterol accumulation in peripheral macrophages, a phenotype similar to that of TD patients [31]. However, atherosclerotic lesions were not increased when $Abca1^{-/-}$ mice were fed chow or a high-fat, high-cholesterol (HF/HC) diet [31]. Upon cross-breeding of $Abca1^{-/-}$ mice with hypercholesterolemic mouse models ($apoE^{-/-}$ or $Ldlr^{-/-}$ mice), the accumulation of lipid-laden foam cells in peripheral tissues was especially pronounced [32]. However, compared with the control mice, lesion size was not increased in either $Abca1^{-/-}apoE^{-/-}$ or $Abca1^{-/-}Ldlr^{-/-}$ double knockout mice, which is probably caused by a less atherogenic lipid profile in these animals. Recent studies have used tissue-specific knockout or knockdown of the Abca1 locus to assess the respective role of liver, intestine, and macrophage ABCA1 in HDL metabolism and atherogenesis. Liver-specific $Abca1^{-/-}$ mice have a ≈80% reduction in HDL-C levels [33], and liver-specific partial gene knockdown of Abca1 significantly reduced HDL-C levels by ≈40% [34]. Subsequent studies reported that intestine-specific $Abca1^{-/-}$ mice have a ≈30% reduction in HDL-C levels [35]. Thus, the liver and intestine that synthesize apoA-I [36, 37], are primarily responsible for lipidating newly secreted lipid-poor apoA-I via ABCA1-mediated lipid efflux. More recently, Brunham *et al.* elegantly showed the tissue-specific role of ABCA1 in influencing susceptibility to atherosclerosis [38]. Their results indicated that physiologically global ABCA1 overexpression reduced atherosclerosis. Deletion of hepatic Abca1 significantly accelerated atherosclerosis, indicating that the liver is an important site at which ABCA1 plays an antiatherogenic role. Surprisingly, selective deletion of macrophage ABCA1 was not found to have a strong impact on lesion burden. Moreover, previous studies have shown that, independently of changes in plasma HDL-C levels, deletion of Abca1 in bone marrow-derived hematopoietic cells increases atherosclerosis [32, 39], whereas overexpression of bone marrow ABCA1 reduces atherosclerosis [40]. Whether ABCA1 expression in other hematopoietic cells (e.g., dendritic cells, lymphocytes, and endothelial progenitor cells) prevents atherosclerosis via modulating immunologic responses, including the secretion of inflammatory cytokines and T cell proliferation, remains to be further investigated.

Together these studies demonstrate that ABCA1 is a rate-limiting factor in HDL biosynthesis. In liver and intestine ABCA1 is essential for maintaining plasma HDL-C levels, while macrophage ABCA1 only has a minor effect on circulating HDL but is important as a mediator of cellular lipid efflux to exogenous lipid-poor HDL apolipoproteins (Fig. **1**). Physiological deletion or expression of ABCA1 modulates susceptibility to atherosclerosis in mice, and hepatic ABCA1 may be an appropriate therapeutic target for raising HDL levels and reducing atherosclerosis in humans.

1.2. ABCG1, HDL, and Atherosclerosis

ABCG1, also known as ABC8 or human white gene, exhibits high homology with the Drosophila white gene, which acts as a regulator of tryptophan and guanine uptake in conjunction with the scarlet and brown genes [41, 42]. To date, no genetic variations in human ABCG1 have been identified that link it to any inheritable disease. Nevertheless, Mauldin *et al.* reported that ABCG1 expression and function

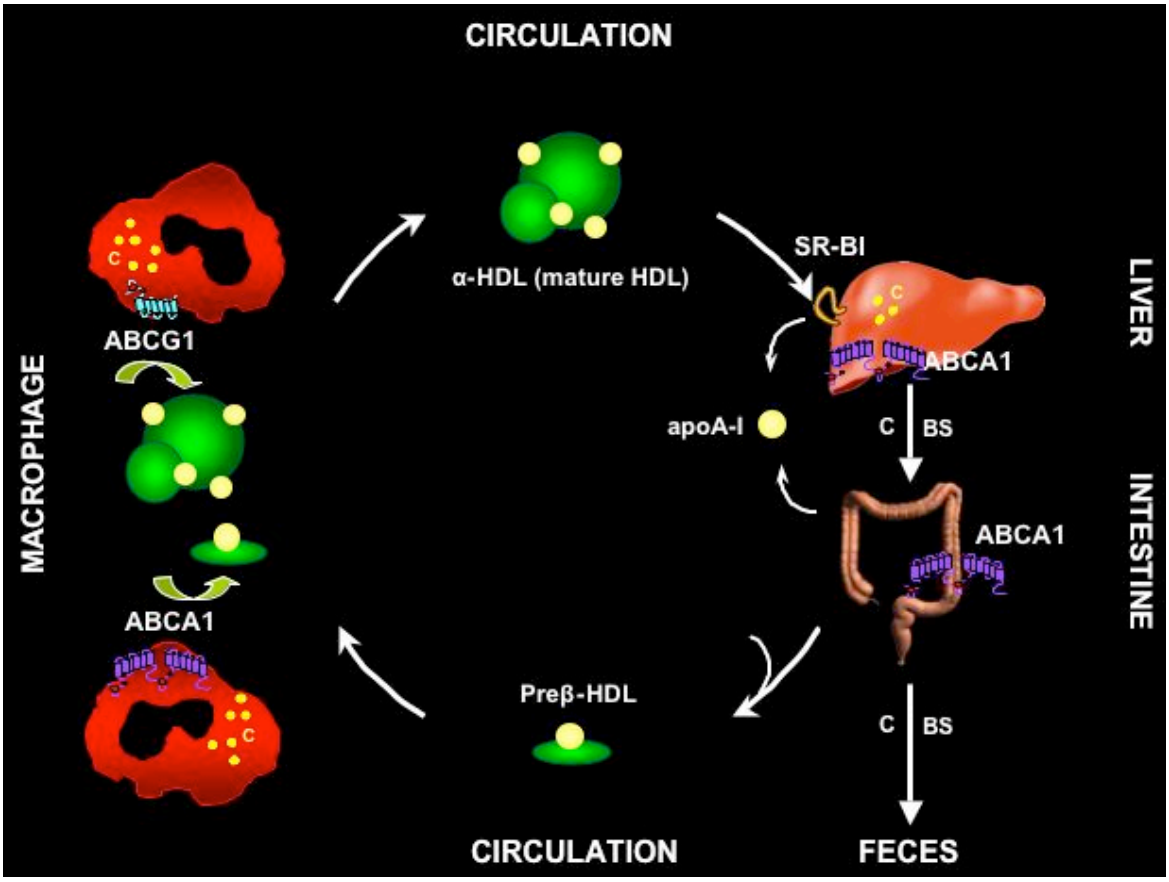


Fig. (1). Schematic overview of the role of ABCA1 and ABCG1 in the reverse cholesterol transport (RCT). apoA-I is secreted by liver and intestine and loaded with cholesterol (C) and phospholipids by ABCA1. The thus formed preβ-HDL picks up cholesterol and phospholipid from ABCA1 in macrophages and peripheral cells and is converted to α-HDL. These mature HDL particles can be further loaded with cholesterol at least in part by ABCG1 in macrophages and delivers in turn its cargo to SR-BI in the liver. Subsequently, cholesterol can be secreted into the bile either in the free form or after conversion as bile salt (BS). After transport via the bile into the intestine, cholesterol and bile salts are reabsorbed or excreted in the feces.

are significantly reduced in patients with type 2 diabetes mellitus, potentially contributing to the formation of lipid-laden macrophages and accelerated atherosclerosis in these patients [43]. Schou *et al.* demonstrated that a functional variant in the ABCG1 promoter, ABCG1 -376C>T located in a putative SP1 binding site, reproducibly predicts an increased incidence of ischemic heart disease in the general population [44]. In addition, Furuyama *et al.* reported that another ABCG1 -257T>G promoter polymorphism confers an appreciable increase in the risk of atherosclerotic disease severity in Japanese men [45]. In the latter two studies, plasma HDL-C levels are relatively normal in subjects with ABCG1 mutations [44, 45]. However, Furuyama et al suggested that ABCG1 may influence the lipid composition in HDL particles, affect the turnover of HDL metabolism and atherogenesis in humans [45]. In an attempt to determine the function for human ABCG1, Klucken *et al.* treated human macrophages with antisense oligonucleotides to ABCG1, and this treatment resulted in decreased efflux of cholesterol and phospholipids to HDL3 [7]. However, the exact cellular and physiological function of ABCG1 remained unclear.

Studies evaluating the physiological relevance of ABCG1 transport activity have relied mainly on mouse models where the endogenous Abcg1 locus is deleted or human ABCG1 is overexpressed. Kennedy *et al.* first reported that Abcg1−/− animals did not display an overt dyslipidemia when maintained on a chow diet [46]. However, these null animals accumulated massive both neutral lipids and phospholipids in hepatocytes and in macrophages within multiple tissues following administration of a HF/HC diet. Conversely, overexpression of human ABCG1 protected murine tissues from dietary fat-induced lipid accumulation. Despite these observations, when administered a HF/HC diet, loss of total body Abcg1 transport did not significantly accelerate atherosclerosis, and loss of macrophage Abcg1 in atherosclerotic mouse models ($apoE^{-/-}$ or $Ldlr^{-/-}$ mice) has been variously reported to be either pro- or antiatherosclerotic [47-50]. Moreover, total body human ABCG1 overexpression resulted in either no effect [51] or aggravation [52] of atherosclerosis. These results indicate that manipulating ABCG1 alone does not markedly impact atherosclerosis in mice.

ABCA1 converts lipid-poor/free apoA-I to partially lipidated "nascent" HDL, and these particles in turn may serve as substrates for ABCG1-mediated cholesterol export [53]. This raises the interesting possibility that ABCA1 and ABCG1 may coordinate the removal of excessive cellular cholesterol. Recently, Abca1 and Abcg1 double knockout ($Abca1^{-/-}Abcg1^{-/-}$) mice were generated by several

independent groups to investigate the potential synergistic relationship between ABCA1 and ABCG1 in modulating macrophage cholesterol homeostasis and atherogenesis. Out *et al.* reported that combined Abca1 and Abcg1 deficiency resulted in a completely abolished cholesterol mass efflux from macrophages to HDL [54]. Similar studies by Yvan-Charvet *et al.* showed that $Abca1^{-/-}Abcg1^{-/-}$ macrophages displayed a more than 2-fold decrease in the ability to promote cholesterol efflux to HDL or serum as compared to $Abca1^{-/-}$, $Abcg1^{-/-}$ and wild-type macrophages [55]. *In vivo* studies using a macrophage-specific RCT assay also indicated that cholesterol efflux from $Abca1^{-/-}Abcg1^{-/-}$ macrophages to plasma, liver, and feces was highly reduced as compared to animals that received macrophages from single knockout or wild-type mice [56]. Defective cholesterol efflux due to the combined deficiency of Abca1 and Abcg1 was associated with markedly increased cholesterol content, primarily in the form of cholesteryl ester (CE), in resident macrophages of the peritoneal cavity, lung, liver, spleen, Peyer's patches, and lymph nodes in chow-fed $Abca1^{-/-}$ $Abcg1^{-/-}$ mice [54]. However, these mice did not show lipid accumulation in the arterial wall. This is most likely caused by the severe hypocholesterolemia in these mice, which is unable to provide the stimulus to attract macrophages to infiltrate into the arterial wall. Furthermore, Yvan-Charvet *et al.* showed that $Ldlr^{+/-}$ mice receiving $Abca1^{-/-}Abcg1^{-/-}$ transplants had substantially greater atherosclerosis as compared to recipients receiving single knockout or wild-type bone marrow, following administration of a HF/HC bile salt diet (1.25% cholesterol, 7.5% cocoa butter and 0.5% cholic acid) [55]. Out *et al.* reported that reconstitution of $Ldlr^{-/-}$ mice with $Abca1^{-/-}Abcg1^{-/-}$ bone marrow cells resulted in a prominent accumulation of lipids in the liver, lung and spleen as compared to recipients receiving single knockout or wild-type bone marrow, following a Western-type diet (0.15% cholesterol and 15% fat) [57]. However, in the context of $Ldlr^{-/-}$ recipients, loss of ABCG1 did not significantly worsen atherosclerosis beyond that caused by the loss of only ABCA1, which is possibly reflecting an unexpected decreased in plasma VLDL/LDL levels in the $Abca1^{-/-}Abcg1^{-/-}$ transplanted mice. The study of Out *et al.* was interpreted as showing a disproportionate increase in atherosclerosis given the degree of VLDL/LDL lowering.

Taken together, these studies indicate that ABCG1 plays a critical role in lipid homeostasis by controlling tissue lipid levels and the efflux of cellular cholesterol to HDL (Fig. **1**). Unlike ABCA1, ABCG1 does not have a rate-limiting role in maintaining plasma HDL-C levels. Likewise, loss of ABCG1 alone does not markedly accelerate atherosclerosis. In spite of having a varied effect on atherosclerosis, ABCG1 in concert with ABCA1 does make a major contribution to cellular cholesterol export and prevent macrophage foam cell formation.

2. ABCA1 AND ABCG1 IN INFLAMMATION, INDEPENDENT OF CHANGES IN CIRCULATING HDL LEVELS

Atherosclerosis is a chronic inflammatory disease in which macrophages and endothelial cells (EC) play important roles. Macrophages are a primary cell type involved in innate immunity. Lipopolysaccharide (LPS), a major outer membrane constituent of Gram-negative bacteria, is a potent endotoxin that, through the activation of cellular immunity, induces a cytokine-mediated systemic inflammatory response in the host [58, 59]. A series of studies have shown that deficiency of ABCA1 and/or ABCG1 in macrophages leads to LPS hypersensitivity [55, 60, 68, 78], implying a potential role of these two transporters in suppressing macrophage inflammation. In addition to macrophages, aortic EC also express ABCA1 and ABCG1 and export cholesterol to HDL [61]. EC in the artery wall become activated in many cases by oxidized lipids in early atherogenesis [62]. Activated EC secrete pro-inflammatory chemokines that recruit monocytes to the activated endothelium [63]. Several studies have shown that incubation of EC with HDL reduces the endothelial inflammatory response [64-66]. It is thus possible that ABCA1 and ABCG1 modulate inflammatory responses in vascular EC at least in part by facilitating cholesterol efflux to HDL in these cells.

2.1. Anti-inflammatory Properties of ABCA1

Francone *et al.* reported that LPS-induced septic shock was exacerbated in $Abca1^{-/-}Ldlr^{-/-}$ mice compared with $Ldlr^{-/-}$ mice [59], suggesting a potential role of ABCA1 in suppressing inflammation. Subsequent studies by Aiello *et al.* showed that macrophages from $Abca1^{-/-}Ldlr^{-/-}$ mice were enriched in CE content (80-fold higher), and plasma HDL concentrations in these mice were extremely low [67]. However, it is not clear whether the observed heightened response to LPS in macrophages from $Abca1^{-/-}Ldlr^{-/-}$ mice is due to the massive cellular CE accumulation, the low plasma HDL-C levels, or some other alterations. Recently, macrophage-specific $Abca1^{-/-}$ mice were generated by Zhu *et al.* [68], and it was demonstrated that macrophages lacking Abca1 are hypersensitive to LPS stimulation, independently of low plasma HDL-C levels, requiring the expression of MyD88 which is an adaptor protein that mediates LPS signaling initiated by the Toll-like receptor 4 (TLR4). Koseki *et al.* showed that cultured Abca1−/− macrophages secreted greater amounts of pro-inflammatory tumor necrosis factor-α (TNF-α) in response to LPS, and that this response was associated with increased levels of free cholesterol (FC) in lipid rafts on the cell surface [69]. These data support the concept that ABCA1 modulates cell surface cholesterol levels and inhibits its partitioning into lipid rafts, which may explain the hypersensitivity to LPS in $Abca1^{-/-}$ macrophage. Moreover, Sun *et al.* reported that FC accumulation in the endosomal compartment increased the inflammatory response in a TLR-dependent fashion, with TLR3 playing the major role [70]. Combined, it is plausible that the anti-inflammatory properties of ABCA1 are mediated by its ability to modulate cholesterol content both on the cell surface and within intracellular compartments (Fig. **2**).

However, recent work by Tang *et al.* suggested that not all of the anti-inflammatory properties of ABCA1 are a consequence of its lipid transport activity [71]. It was reported that the interaction of apoA-I and its mimetic peptides with ABCA1 promotes cholesterol removal and activates signaling molecules, such as Janis kinase 2 (JAK2), which optimize the lipid export activity of ABCA1 [72, 73]. JAK2 activation, however, also stimulates signaling pathways that activate transcription factors called STATs [74]. One of the

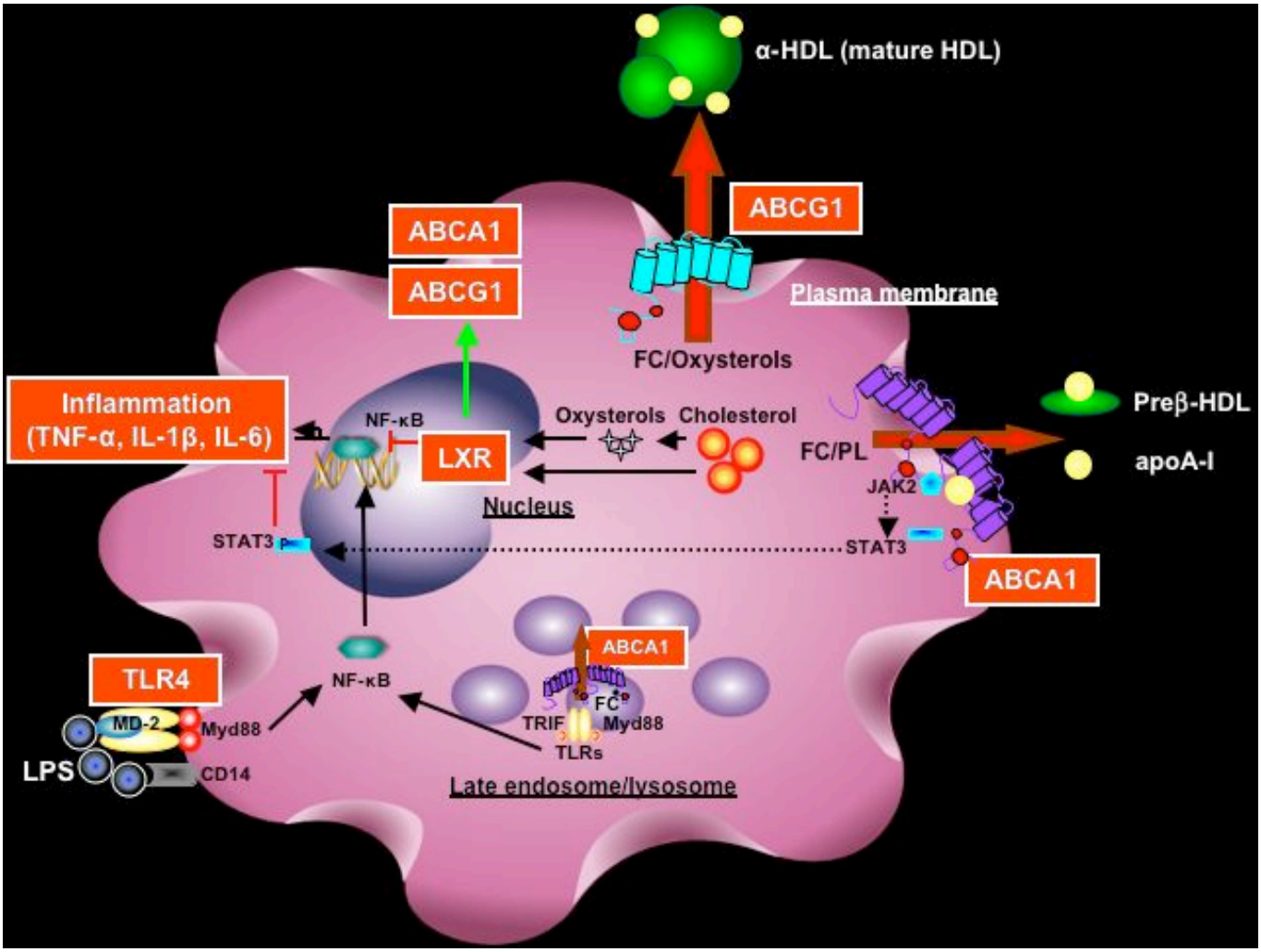


Fig. (2). Protective effects of ABCA1 and ABCG1 on macrophage cholesterol efflux and inflammation. The ABC transporters ABCA1 and ABCG1 coordinate to facilitate macrophage cholesterol efflux to HDL and its apolipoproteins. ABCA1 transports phospholipids (PL) and cholesterol, while ABCG1 is largely a cholesterol transporter. Cholesterol efflux to apoA-I specifically requires ABCA1, whereas efflux to HDL requires ABCG1. Unlike ABCA1, ABCG1 has a specific role in promoting cellular efflux of oxysterols modified at the 7 position, such as 7-ketocholesterol- and 7β-hydroxycholesterol. Activation of LXR by oxysterols robustly induces the transcriptional expression of ABCA1 and ABCG1 in macrophages, leading to enhanced cholesterol efflux and resolution of the cholesterol overload in these cells. ABCA1 and ABCG1 modulate the fluidity of the plasma membrane as well lipid raft formation. This, in turn, modulates the Toll-like receptor 4 (TLR4) expression and signaling upon activation via the LPS/MD-2 complex formed after binding of LPS to CD14. Subsequently, downstream signaling molecules such as Myd88 will control the activation of NF-κB and the inflammatory gene expression response. Activation of LXR can also modulate the inflammatory response through suppression of NF-κB, but this mechanism appears to be independent of ABCA1 or ABCG1 activity. Free cholesterol (FC) accumulation in the late endosome/lysosome can also modulate the inflammatory response by spontaneous activation of TLRs, such as TRIF-dependent TLR3, present in these intracellular compartments. By promoting cholesterol efflux from the late endosomal compartment, ABCA1 thus have a unique role in dampening inflammation. In addition, binding of apoA-I to ABCA1 stimulates the activation of signaling molecules, such as Janis kinase 2 (JAK2). The activated JAK2 both enhances the interaction of apoA-I with ABCA1 and stimulates the transcription factor STAT3. The phosphorylated STAT3 is translocated to the nucleus where it suppresses LPS-mediated production of inflammatory cytokines, without inhibiting LPS-induced NF-κB activation.

STATs, STAT3, plays a major role in suppressing inflammatory cytokine production by macrophages [74, 75]. As a result, incubation of apoA-I with activated ABCA1-expressing macrophages suppressed the production of inflammatory cytokines TNF-α, IL-1β, and IL-6 by a STAT3-dependent process [75]. This novel role of ABCA1 was further confirmed by results showing that apoA-I lost its anti-inflammatory activity in Abca1$^{-/-}$ macrophages [75]. The generation of mutant forms of ABCA1 that lack the ability to activate STAT3 but retain lipid export activity is worth exploiting, and is expected to provide important mechanistic insight into the anti-inflammatory properties of ABCA1 (Fig. **2**).

2.2. Anti-inflammatory Properties of ABCG1

Wojcik *et al.* reported that Abcg1$^{-/-}$ mice had increased pro-inflammatory cytokine levels in their lungs, leading to the recruitment of neutrophils, eosinophils, B cells, T cells, and dendritic cells [76]. They also showed that macrophages

are the primary cell type involved in the onset of both pulmonary lipidosis and inflammation in Abcg1$^{-/-}$ mice. Likewise, Yvan-Charvet *et al.* found that macrophages lacking either Abcg1, or both abca1 and abcg1 secreted significantly more cytokines and chemokines [55]. Both ABCA1 and ABCG1 modulate cell surface cholesterol levels and inhibit its partitioning into lipid rafts. Given lipid rafts may provide platforms for innate immune receptors to respond to inflammatory signals [77], it follows that loss of ABCA1 and ABCG1 by increasing raft content will increase signaling through these receptors. In agreement, basal and LPS-stimulated thioglycollate-elicited peritoneal macrophages showed increased inflammatory gene expression in the order Abca1$^{-/-}$Abcg1$^{-/-}$>Abcg1$^{-/-}$>Abca1$^{-/-}$>wild-type, and TLR4 cell surface concentration was increased in Abca1$^{-/-}$Abcg1$^{-/-}$>Abcg1$^{-/-}$>Abca1$^{-/-}$>wild-type macrophages [78]. Interestingly, replenishment or removal of cholesterol using cyclodextrin reduced the inflammatory response of Abca1$^{-/-}$ and Abcg1$^{-/-}$ macrophages [78, 79], supporting the hypothesis that the increased inflammatory response is attributable to cholesterol accumulation in the plasma membrane of Abca1$^{-/-}$ and Abcg1$^{-/-}$ macrophages. ABCG1 appears to have a more potent role in modulating macrophage inflammation than ABCA1, which perhaps reflects a predominant role of this transporter in modulating cholesterol content in lipid rafts [80].

Both apoptosis and necrosis was increased in Abcg1$^{-/-}$ alveolar macrophages [76], which may also contribute to or dampen pulmonary inflammation. In agreement, Baldán *et al*. showed that Abcg1$^{-/-}$ peritoneal macrophages were more apoptotic [47]. When FC accumulates in the macrophage, the ratio of FC to phospholipids is disturbed in the endoplasmic reticulum (ER) membrane [81]. The "stiffening" of the ER membrane bilayer leads to ER protein dysfunction, ER stress, and the unfolded protein response, resulting in apoptosis of the cell [81]. Furthermore, oxysterols that are formed within macrophages as a consequence of cholesterol accumulation may cause cells to be more prone to apoptosis [82, 83]. Unlike ABCA1, ABCG1 has a specific role in promoting cellular efflux of oxysterols modified at the 7 position, such as 7-ketocholesterol- and 7β-hydroxycholesterol, to prevent macrophages from oxysterol-induced cell death [84, 85]. Thus, it is possible that Abcg1$^{-/-}$ macrophages are more prone to apoptosis due to the toxic effects of FC and oxysterol accumulation.

More recently, Whetzel *et al.* reported that reductions in ABCG1 expression in endothelium promote a pro-inflammatory endothelial phenotype [86]. Aortic EC from Abcg1$^{-/-}$ mice displayed increased production of chemokines, increased surface expression of the adhesion molecules that promote monocyte adhesion and interactions. The regulation of monocyte-endothelial interactions by ABCG1 appeared to be independent of HDL binding. Furthermore, the pro-inflammatory phenotype of Abcg1$^{-/-}$ EC was not mediated by NFκB, a key molecule involved in the regulation of the inflammatory response to both infection and tissue damage. Instead, the IL-6-IL-6 receptor axis was found to be induced in Abcg1$^{-/-}$ EC, and this pathway, through STAT3 signaling, regulates monocyte-endothelial interactions in Abcg1$^{-/-}$ mice. Thus, in addition to suppressing macrophage inflammation, ABCG1 may also be important for regulating the early inflammatory response in EC in the vessel wall. Further studies examining whether the effect of ABCG1 deficiency on the total cholesterol content and/or the intracellular distribution of cholesterol in EC as well as macrophages and the consequence for cellular signaling are needed to fully understand the anti-inflammatory properties of ABCG1.

3. STRUCTURE, EXPRESSION AND REGULATION OF ABCA1 AND ABCG1

3.1. The Structure, Expression, and Regulation of ABCA1

ABCA1 is a 2261 amino acid integral membrane protein. It is a full transporter, comprising two halves of similar structure. Each half has a transmembrane domain containing six helices and a nucleotide binding domain (NBD) containing two conserved peptide motifs known as Walker A and Walker B, which are present in many proteins that use ATP in energizing their transport activity, and a Walker C signature unique to ABC transporters. ABCA1 is predicted to have an N terminus oriented into the cytosol and two large extracellular loops that are highly glycosylated and linked by one or more cysteine bonds [87, 88].

ABCA1 is expressed ubiquitously, but with the highest concentrations in the liver, brain, adrenal glands, and macrophage foam cells [89-91]. Experiments using biotinylation [92], anti-FLAG antibody in FLAG-ABCA1 transfected cells [93], green fluorescent protein (GFP)-ABCA1 transfected cells [94, 95], and a polyclonal antibody [96] have suggested that ABCA1 localizes to the plasma membrane and to intracellular compartments (e.g., early and late endosomes and lysosomes). Although still controversial, it is suggested that ABCA1 may form a channel in the plasma membrane that promotes flipping of lipids from the inner to outer membrane leaflet by an ATPase-dependent process [97]. Some data support a direct efflux mechanism wherein ABCA1 first binds apoA-I, resulting in subsequent transfer of lipid to the bound apoA-I, which is then released from the cell, with the latter steps presumably still occurring within the context of the high affinity complex [98, 99]. Other more indirect models have also proposed that ABCA1 activity creates lipid raft membrane domains in the absence of apoA-I, and that these lipid domains may represent a larger capacity but lower affinity cellular binding site for apoA-I that is not closely associated with ABCA1 [100, 101]. Subsequently, cellular phospholipids and cholesterol are transported simultaneously or phospholipid efflux precedes cholesterol efflux [102, 103]. These partially lipidated apolipoproteins can then acquire additional cholesterol by other processes and mature into larger spherical HDL particles (HDL2 and HDL3). The selective uptake of HDL by the liver is mediated by scavenger receptor class B type I (SR-BI), which has a high affinity for lipid-rich HDL particles, but low affinity for lipid-poor HDL apolipoproteins [104].

In addition to mediating lipid efflux on the cell surface, ABCA1 may also be internalized along with apoA-I in vesicles to intracellular compartments where ABCA1 pumps lipids into the vesicles for association with apoA-I, and subsequent release of nascent HDL particles upon fusion with the plasma membrane (retroendocytosis) [105, 106]. Smith *et al.* reported the uptake and resecretion of labeled

apoA-I [107] and colocalization of GFP-ABCA1 and apoA-I in intracellular compartments [108]. In agreement, Hassan *et al.* showed that cell binding of apoA-I specifically mediates the continuous endocytic recycling of ABCA1, and that this pathway plays a central role in the genesis of nascent HDL [109]. Moreover, Chen *et al.* found impaired ABCA1 internalization in cells expressing a mutant form of ABCA1 (ABCA1delPEST), and the failure of the ABCA1-PEST mutant to reach late endosomes was associated with less ability to export endosome/lysosomal cholesterol to apoA-I, but not cell surface cholesterol [110]. They also reported that trafficking of ABCA1, and possibly apoA-I, to the late endosome/lysosome compartment is responsible for a quantitatively significant percentage of total ABCA1-dependent cholesterol efflux [110]. Thus, it is conceivable that internalization of ABCA1 is necessary for the mobilization of cellular cholesterol, at least during transit through the late endosome/lysosome compartment.

As expected for a transporter that mediates excess cellular cholesterol export, transcription of ABCA1 is markedly induced by overloading cells with cholesterol [89, 90]. ABCA1 expression is regulated at multiple levels, including the nuclear receptors liver X receptor (LXR) and retinoid X receptor (RXR) binding to the promoter region of the ABCA1 gene, with LXR being activated by elevated levels of oxysterols in cholesterol-loaded cells [111, 112]. Several additional modes of transcriptional regulation of ABCA1 by the transcription factors Sp1/3, upstream stimulatory factor 1/2 (USF1/2), hepatocyte nuclear factor 1a (HNF-1a), cyclic AMP (cAMP), and Zinc finger protein 202 (ZNF202) have been reviewed [113]. Although much emphasis has been placed on the transcriptional regulation of ABCA1, new studies are beginning to reveal its post-transcriptional regulation. Under basal conditions in cultured cells, ABCA1 has a relatively rapid turnover rate, as evidenced by the fact that ABCA1 protein is highly unstable in absence of inducers (half-life of 1 to 2 hours) [114-116]. ABCA1 is stabilized by apoA-I binding to cells, through apoA-I-dependent inhibition of calpain-mediated proteolysis of ABCA1 [115]. Multiple constitutive and apoA-I-stimulated phosphorylation events have been described that both enhance [72, 117-121] or inhibit [122] ABCA1 activity. In addition, Arakawa and Yokoyama showed that apoA-I itself stabilizes ABCA1 by retarding its degradation [123]. In their study, A,N-acetyl-leucine-leucyl-norleucinal aldehyde (ALLN), but not lactacystin, blocked the turnover of ABCA1 in the absence of apoA-I, and these authors speculated that a non-proteasomal thiol protease was involved. In another study by Feng et al., the decrease in ABCA1 protein in FC-loaded macrophages was blocked to similar degrees by ALLN and lactacystin, suggesting an important role for proteasomal degradation in this process [124]. An important area of future investigation will be to determine the molecular mechanisms linking FC loading to proteasomal degradation of ABCA1, including the possibility that FC loading triggers ubiquitination of ABCA1. Moreover, Wang and Oram reported that unsaturated fatty acids (FAs) also accelerate the degradation of ABCA1 and thus decrease apoA-I-mediated efflux [116]. Unsaturated FAs not only act on the transcriptional regulation of ABCA1 (as antagonists of oxysterols in LXR activation), but also induce ABCA1 protein degradation (via a signaling pathway involving activation of phospholipase D2 and phosphorylation of ABCA1 serines) [125].

3.2. The Structure, Expression, and Regulation of ABCG1

ABCG1 is a half-transporter containing only one 6-helix transmembrane domain and a single nucleotide binding fold. As an active transporter which needs two NBD and two transmembrane bundles, ABCG1 needs to form a homodimer or a heterodimer with another ABC transporter to become active [126, 127].

In general, high expression of ABCG1 mRNA was noted in spleen, lung, thymus, and brain, whereas expression in kidney, liver, and heart was low or undetectable [127, 128]. Subsequent studies using ABCG1-null/LacZ knock-in mice demonstrated that ABCG1 is predominantly expressed in macrophages, EC, and lymphocytes [46]. The mammalian ABCG1 cDNA was originally identified from studies using degenerate PCR and RNA from either a murine macrophage cell line [127], a murine pre-B cell library [128], or a human Jurkat T-cell line [128], or after using exon-trapping and a human chromosome 21 cDNA library [129]. Both the human and murine ABCG1 genes utilize multiple promoters and alternative splicing to produce a diverse array of mRNAs that encode multiple putative protein isoforms that range from 64-79 kDa [130-132]. It is not known if these isoforms, which vary only at the amino terminus upstream of the Walker A motif, form distinct dimers and/or have different functions *in vivo*. One interesting human isoform variation is predicted to arise due to alternative splicing in a section of the cytoplasmic domain of ABCG1 between the transmembrane regions and the ATP cassette. Splicing in this region leads to either the presence or absence of a 12 amino acid (AA) internal segment in the ABCG1 protein, termed as ABCG1(+12) and ABCG1(−12) [128, 133]. The two ABCG1 isoforms are expressed at both RNA and protein levels in human macrophages, with ABCG1(−12) tending to be the more abundant [134]. Of note, ABCG1(+12) is not expressed in mice [135], while mouse models are widely used to elucidate the function of ABCG1, further investigations into the importance of this human ABCG1 isoform are warranted.

ABCG1 promotes cholesterol efflux from cells to mature HDL but not to lipid-free apoA-I [7, 136, 137]. In contrast to ABCA1-dependent lipid export, which requires apolipoprotein binding, ABCG1-dependent cholesterol efflux does not appear to directly bind HDL [138]. Different models have been proposed to explain how ABCG1 acts to increase the availability of plasma membrane cholesterol to its acceptors. One suggested that ABCG1 helps cholesterol molecules to overcome the energy barrier for entry into the hydrophilic water layer, perhaps by using ATP to promote protrusion of the cholesterol molecule into water, followed by a transient collision with an acceptor [138]. A second model pointed to a function of ABCG1 as a phospholipid floppase, promoting changes in the organization of plasma membrane phospholipids and subsequent attraction of cholesterol to the outer leaflet for diffusional efflux [139]. Although expression of ABCG1 does not increase cellular binding of HDL, it does increase the oxidation sensitivity of cellular cholesterol in the absence of HDL [100, 137], suggesting that ABCG1

(like ABCA1) can modify plasma membrane cholesterol domains such that they are more accessible to cholesterol oxidase. Whether the cholesterol in the ABCG1 modified membrane domains is spatially different from those generated by ABCA1 remains to be investigated.

ABCG1 predominantly localizes to the intracellular compartments in basal macrophages, and it can be redistributed to the plasma membrane after LXR activation [140]. Thus, the LXR system performs a dual function of inducing ABCG1 transcription and its translocation to the plasma membrane. However, one recent study by Larrede *et al.* demonstrated that stimulation of cholesterol efflux to HDL by LXR activation in human foam cells involves an ATP-dependent transport mechanism solely mediated by ABCA1 that it appears to be independent of ABCG1 expression [141]. In addition to LXR activation, ABCG1 expression can also be induced by agonist of peroxisome proliferator-activated receptor gamma (PPARγ) paralleling with increased LXR [142]. In agreement, Akiyama *et al.* showed that hepatic expression of ABCG1 was reduced in mice following conditional disruption of hepatic PPARγ [143]. Moreover, PPARγ agonists were reported to induce ABCG1 in LXR α/β-null macrophages and to exert antiatherogenic effects [144]. These results suggest that the expression of macrophage ABCG1 could be increased by PPARγ agonists, which is associated with or independent of LXR activation.

Like ABCA1, ABCG1 expression can also be post-transcriptionally regulated by diverse factors. Uehara *et al.* demonstrated that unsaturated FAs significantly suppress the stimulatory effects of oxysterols and retinoids on the mRNA and protein expression of ABCG1 as well as the activity of the wild-type human ABCG1 promoter [145]. In addition, lipoxygenases comprise a family of enzymes capable of mediating selective lipid oxidation. One of them, called 12/15-lipoxygenase (12/15LO) catalyzes the conversion of free FAs to produce eicosanoids [146]. Nagelin *et al.* previously showed that 12/15LO activity in macrophages enhances the degradation of ABCG1 protein and thus reduced cholesterol efflux [147]. Correlating with the increased turnover of ABCG1, the 12/15LO eicosanoid product 12S-hydroxyeicosatetranoic acid (12SHETE) enhances the serine phosphorylation of ABCG1 [147]. More recently, the mechanism by which 12/15LO regulates ABCG1 was further elucidated by the same group, and it was suggested that 12/15LO activates c-Jun NH2-terminal kinase 2 (JNK2) and p38 mitogen-activated protein kinase (MAPK) pathways to enhance the phosphorylation and degradation of ABCG1 [148]. Furthermore, Mauldin *et al.* demonstrated that ABCG1 expression was decreased in peritoneal macrophages isolated from Type 2 diabetic mice [149]. Similarly, down-regulation of ABCG1 was observed by using C57BL/6J peritoneal macrophages cultured in the presence of elevated glucose levels. These results suggest that ABCG1 expression in diabetic macrophages is regulated by chronic exposure to elevated glucose. Zhou *et al.* recently reported that the expression of ABCG1, but not ABCA1, was reduced in monocytes from Type 2 diabetic patients, which is partly related to the increased exposure to serum advanced glycation end (AGE) products [150]. The effect of AGEs on ABCG1 expression is mediated by the receptor for AGE (RAGE) through a LXR-independent pathway. However, the exact mechanism remains unknown [151].

4. THERAPEUTIC TARGETING ABCA1 AND ABCG1

As described above ABCA1, but not ABCG1, is a rate-limiting factor in HDL biosynthesis. Both ABCA1 and ABCG1 play critical roles in lipid homeostasis by controlling tissue lipid levels and cellular cholesterol efflux. Cholesterol efflux to apoA-I specifically requires ABCA1, whereas efflux to HDL requires ABCG1. In addition, ABCA1 and ABCG1 have also anti-inflammatory properties which are independent of changes in plasma HDL-C levels. The beneficial effects of ABCA1 and ABCG1 have made them important new therapeutic targets for preventing atherosclerosis. The expression and activity of ABCA1 and ABCG1 is regulated transcriptionally and post-transcriptionally by multiple processes. Nowadays, various programs have been initiated by the pharmaceutical industry to target these two ABC transporters.

4.1 Target Transcription of ABCA1 and ABCG1

Both ABCA1 and ABCG1 are strongly induced in human macrophages on LXR/RXR activation, and ABCG1 appears to be a preferential target of LXR ligands in foam cells [141]. Thus, upregulation of macrophage ABCA1 and ABCG1 by LXR agonists may constitute an effective pharmacological approach to attenuate foam cell formation, and thereby to prevent arterial lipid accumulation and lesion progression in dyslipidemic patients. In addition to ABCA1 and ABCG1, LXR agonists are also known to induce the transcription of other molecules involved in the RCT pathway, including apoE [152], which promotes cholesterol efflux from macrophages by both ABCA1-independent and -dependent mechanisms; liver and intestinal ABCG5 and ABCG8 [153], which promote excretion of liver and dietary cholesterol into the bile; lipid transfer proteins, such as cholesteryl ester transfer protein (CETP) [154] and phospholipid transfer protein (PLTP) [155], which remodel extracellular HDL particles to regenerate lipid-free apolipoproteins. On the other hand, Joseph *et al.* reported that LXR is important for macrophage survival and regulation of the innate immune response, but this mechanism does not seem to be controlled by ABCA1 or ABCG1 activity [156]. In agreement, Valledor *et al.* demonstrated that LXR and RXR coordinately regulate the network of genes that control programmed cell death, resulting in protection of macrophages from bacterial-induced apoptosis [157].

Initial studies using two non-steroidal synthetic LXR agonists (TO901317 and GW3965) in mice suggested they were able to increase plasma HDL-C levels and reduce atherosclerosis [158-161]. Recently, Verschuren *et al.* demonstrated that TO901317 strongly suppressed atherosclerotic lesion evolution and promoted lesion regression in apoE*3Leiden mice, a model for atherosclerosis with predictive value for the human situation [162]. Remarkably, the effect of T0901317 on plaque regression occurred even in the presence of proatherogenic plasma cholesterol and triglyceride profiles. The ability of T0901317 to induce atherosclerosis regression is mechanistically linked to (1) enhanced cholesterol efflux from macrophages in the aortic lesions (via upregulation of ABCA1, ABCG1, and apoE); (2) suppressed endothelial monocyte adhesion (at least in part via upregulation of ABCG1); (3) reduced lesional macrophage content by apoptosis; and (4) increased expression of

the chemokine receptor CCR7 in foam cells, which is essential for the emigration of mature dendritic cells to lymph nodes during regression. The exciting possibilities for LXR agonists as new therapeutics are, however, counterbalanced by one significant side effect: the induction of liver de novo lipogenesis and elevation of plasma triglyceride levels [163-165]. This liability is, at least in part, mediated by LXR-induced transcription of sterol regulatory element binding protein-1c (SREBP-1c), which induces some of the key enzymes involved in fatty acid synthesis, such as fatty acid synthase (FAS) and acetyl-coenzyme A carboxylase (ACC) [163, 164].

Clearly, a major challenge for LXR agonists is to achieve a favorable balance between the beneficial effects of induction of cholesterol exporters (e.g., ABCA1 and ABCG1) and the detrimental effects of induction of SREBP-1c. A few possible strategies have been proposed to minimize the side effects of LXR agonist treatment. Both LXR isoforms (i.e., LXRα and LXRβ) respond to the same natural and synthetic ligands, but they show different tissue distribution. LXRα predominates in tissues that synthesize triglycerides, such as the liver, whereas LXRβ has a ubiquitous tissue distribution [166, 167]. It is thus possible that LXR subtypes can control different genes due to their different tissue distribution. Interestingly, peritoneal macrophages from LXRβ knockout mice, but not LXRα knockout mice, show altered basal expression of ABCA1 mRNA [166], suggesting that LXRβ is the subtype more responsible for controlling ABCA1 transcription in macrophages. Furthermore, unlike LXRα-selective agonists, LXRβ-selective agonists may not induce hepatic lipogenesis [167, 168]. However, the crystal structures of LXRα and β are very similar in the ligand binding domains, and it may be a challenge to develop subtype-selective ligands [169, 170]. Another approach is the identification of target gene-selective or tissue-selective LXR agonists. Encouragingly, several natural or synthetic steroidal LXR agonists have been shown to selectively activate gene expression with minimal SREBP-1c gene activation in the liver and strongly induce ABCA1 expression in macrophages [171, 172]. More recently, a novel synthetic steroidal LXR agonist ATI-829 was reported in mice to selectively activate LXR target gene expression in the intestine and macrophages rather than in the liver, without inducing hypertriglyceridemia and liver steatosis [173]. Given the variety of factors that modulate atherosclerosis in mice, the development of LXR agonists that are LXRβ, gene, or tissue selective, which can raise circulating HDL-C and stimulate macrophage cholesterol efflux without causing liver and plasma triglyceride accumulation is critical for the development of LXR-based agonists as therapeutically useful agents.

4.2 Target Post-Transcription of ABCA1 and ABCG1

The possibility of targeting posttranscriptional regulation of ABCA1 and ABCG1 has received less attention. A series of studies have indicated that both ABCA1 and ABCG1 are highly unstable proteins, and that they may be post-transcriptionally regulated *in vivo* by metabolic factors associated with common disorders, such as diabetes. It is also likely that transcription of ABCA1 and ABCG1 in highly cholesterol-loaded cells may already be maximally stimulated. Under these conditions, stimulating transcriptional process alone may have a limited contribution to enhance the overall activity of ABCA1 and ABCG1.

ABCA1 is degraded by calpain [115], and it appears to be one of the major mechanisms for regulation of its cellular level, and helical apolipoproteins protect ABCA1 against this degradation [123]. Helical apolipoproteins interact with ABCA1 and generate new HDL by removing cellular lipid. ABCA1 is phosphorylated and stabilized by a mechanism involving protein kinase C that is presumably activated by diacylglycerol generated by replenishment reaction for the removal of sphingomyeline [120]. To date, several synthetic amphipathic α-helical peptides have been produced. One of them, called D4-F because it contains 4 phenylalanines and only D-amino acids, can be absorbed orally into the blood stream with a low rate of turnover in mice [174]. Administration of this peptide in drinking water has been shown to markedly reduce atherosclerosis in mouse models [174, 175]. D-4F protects against atherosclerosis by several possible mechanisms including interactions with ABCA1. Another advantage of synthetic amphipathic α-helical peptides is that they may be relatively resistant to the oxidative damage which has been reported to impair the interaction of apoA-I with ABCA1 [176]. However, a disadvantage of these peptides is that they have non-specific detergent effects on membrane lipids and can accumulate in tissues and damage cells [177, 178]. Further investigation is needed to develop novel synthetic peptides that mimic amphiphilic helical segments of apolipoproteins to specifically remove cellular lipids via the ABCA1 pathway without having detergent-like effects.

4.3 Other Potential Therapeutic Targets

Another potential strategy under investigation is to increase the concentration of acceptors for ABCA1 and ABCG1. ABCA1 transports phospholipids to lipid-poor apoA-I to generate partially lipidated "nascent" HDL, called preβ-HDL. These preβ-HDL particles can acquire additional cholesterol by other processes and then convert into spherical larger HDL, called α-HDL. These mature HDL particles are effective substrates for ABCG1-mediated cholesterol transport [53, 179-180]. In this regard, intravenous infusion of apoA-I or apoA-I mimetic peptides is promising approaches. Infusion of recombinant apoA-IMilano/phospholipid complexes in apoE$^{-/-}$ mice reduced the foam-cell content of arterial lesions within 48 h post infusion [181]. A weekly infusion of recombinant apoA-IMilano/phospholipid complexes in humans for 5 weeks appeared to induce regression of coronary atherosclerosis in a small study [182]. Currently, one intravenously administered apoA-I mimetic peptide, known as the RLT peptide (ETC-642, Esperion Therapeutics Inc., Ann Arbor, Michigan), is being studied in clinical trials [183]. More recently, Sacks *et al.* developed a novel selective delipidation technique by which large HDL in human or monkey plasma was converted into cholesterol-depleted HDL, resembling preβ-HDL and small spherical HDL3 that are active in removing excessive cholesterol from cells [184]. Importantly, treatment with the delipidated plasma tended to reduce diet-induced aortic atherosclerosis in monkeys, suggesting that the selective HDL delipidation could be an attractive therapy for atherosclerosis.

5. CONCLUSIONS

Studies of human disorders and mouse models show that the ABC cholesterol transporters ABCA1 and ABCG1 have a major impact on whole-body lipid homeostasis by a process termed RCT. ABCA1 and ABCG1 work synergistically to remove excess cholesterol from cells (particularly macrophages), and they also play important roles in suppressing inflammation through multiple mechanisms. In humans, ABCA1 mutations can cause a severe HDL-deficiency syndrome characterized by cholesterol deposition in tissue macrophages and prevalent atherosclerosis. Disrupting Abca1 or Abcg1 in mice promotes accumulation of excessive cholesterol in macrophages, and physiological manipulation of ABCA1 expression affects atherogenesis in mouse models. ABCA1 and ABCG1 have complex cellular and regulatory pathways that are far from being completely understood. At present, a number of approaches have been utilized to target ABCA1 and ABCG1 in efforts to raise circulating HDL levels and stimulate lipid flux through the RCT pathway. However, the efficacy and safety of these therapies remains an open question. As we learn more, there will undoubtedly be additional novel therapeutic targets that offer the best opportunity to treat atherosclerosis.

REFERENCE

[1] Rhoads GG, Gulbrandsen CL, Kagan A. Serum lipoproteins and coronary heart disease in a population study of Hawaii Japanese men. N Engl J Med 1976; 294: 293-8.

[2] Glomset JA. The plasma lecithin:cholesterol acyltransferase reaction. J Lipid Res 1968; 9: 155-67.

[3] Navab M, Berliner JA, Subbanagounder G, *et al.* HDL and the inflammatory response induced by LDL-derived oxidized phospholipids. Arterioscler Thromb Vasc Biol 2001; 21: 481-8.

[4] Epand RM, Stafford A, Leon B, *et al.* HDL and apolipoprotein A-I protect erythrocytes against the generation of procoagulant activity. Arterioscler Thromb Vasc Biol 1994; 14: 1775-83.

[5] Cockerill GW, Huehns TY, Weerasinghe A, *et al.* Elevation of plasma high-density lipoprotein concentration reduces interleukin-1-induced expression of E-selectin in an *in vivo* model of acute inflammation. Circulation 2001; 103: 108-12.

[6] Brewer HB, Jr, Santamarina-Fojo S. New insights into the role of the adenosine triphosphate-binding cassette transporters in high-density lipoprotein metabolism and reverse cholesterol transport. Am J Cardiol 2003; 91: 3-11.

[7] Klucken J, Buchler C, Orso E, *et al.* ABCG1 (ABC8), the human homolog of the Drosophila white gene, is a regulator of macrophage cholesterol and phospholipid transport. Proc Natl Acad Sci USA 2000; 97: 817-22.

[8] Oram JF. HDL apolipoproteins and ABCA1: partners in the removal of excess cellular cholesterol. Arterioscler Thromb Vasc Biol 2003; 23: 720-7.

[9] Schmitz G, Langmann T. High-density lipoproteins and ATP-binding cassette transporters as targets for cardiovascular drug therapy. Curr Opin Investig Drugs 2005; 6: 907-19.

[10] Lajoie P, Goetz JG, Dennis JW, Nabi IR. Lattices, rafts, and scaffolds: domain regulation of receptor signaling at the plasma membrane. J Cell Biol 2009; 185: 381-5.

[11] Strauss JF 3rd, Liu P, Christenson LK, Watari H. Sterols and intracellular vesicular trafficking: lessons from the study of NPC1. Steroids 2002; 67: 947-51.

[12] Luciani MF, Denizot F, Savary S, Mattei MG, Chimini G. Cloning of two novel ABC transporters mapping on human chromosome 9. Genomics 1994; 21: 150-9.

[13] Dean M, Hamon Y, Chimini G. The human ATP-binding cassette (ABC) transporter superfamily. J Lipid Res 2001; 42: 1007-17.

[14] Brooks-Wilson A, Marcil M, Clee SM, *et al.* Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. Nat Genet 1999; 22: 336-45.

[15] Bodzioch M, Orso E, Klucken J, *et al.* The gene encoding ATPbinding cassette transporter 1 is mutated in Tangier disease. Nat Genet 1999; 22: 347-51.

[16] Rust S, Rosier M, Funke H, *et al.* Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. Nat Genet 1999; 22: 352-5.

[17] Lawn RM, Wade DP, Garvin MR, *et al.* The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. J Clin Invest 1999; 104: R25-31.

[18] Walter M, Gerdes U, Seedorf U, Assmann G. The high density lipoprotein- and apolipoprotein A-I-induced mobilization of cellular cholesterol is impaired in fibroblasts from Tangier disease subjects. Biochem Biophys Res Commun 1994; 205: 850-6.

[19] Francis GA, Knopp RH, Oram JF. Defective removal of cellular cholesterol and phospholipids by apolipoprotein A-I in Tangier Disease. J Clin Invest 1995; 96: 78-87.

[20] Horowitz BS, Goldberg IJ, Merab J, Vanni TM, Ramakrishnan R, Ginsberg HN. Increased plasma and renal clearance of an exchangeable pool of apolipoprotein A-I in subjects with low levels of high density lipoprotein cholesterol. J Clin Invest 1993; 91: 1743-52.

[21] Kathiresan S, Melander O, Guiducci C, *et al.* Six new loci associated with blood low-density lipoprotein cholesterol, high-density lipoprotein cholesterol or triglycerides in humans. Nat Genet 2008; 40: 189-97.

[22] Willer CJ, Sanna S, Jackson AU, *et al.* Newly identified loci that influence lipid concentrations and risk of coronary artery disease. Nat Genet 2008; 40: 161-9.

[23] Kathiresan S, Melander O, Anevski D, *et al.* Polymorphisms associated with cholesterol and risk of cardiovascular events. N Engl J Med 2008; 358: 1240-9.

[24] Serfaty-Lacrosniere C, Civeira F, Lanzberg A, *et al.* Homozygous Tangier disease and cardiovascular disease. Atherosclerosis 1994; 107: 85-98.

[25] Assmann G, vonEckardstein A, Brewer HJ. Familial high density lioprotein deficiency: Tangier Disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D. The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York 1995; 2053-72.

[26] van Dam MJ, de Groot E, Clee SM, *et al.* Association between increased arterial-wall thickness and impairment in ABCA1-driven cholesterol efflux: an observational study. Lancet 2002; 359: 37-42.

[27] Frikke-Schmidt R, Nordestgaard BG, Jensen GB, Steffensen R, Tybjaerg-Hansen. A genetic variation in ABCA1 predicts ischemic heart disease in the general population. Arterioscler Thromb Vasc Biol 2008; 28: 180-6.

[28] Frikke-Schmidt R, Nordestgaard BG, Stene MC, *et al.* Association of loss-of-function mutations in the ABCA1 gene with high-density lipoprotein cholesterol levels and risk of ischemic heart disease. JAMA 2008; 299: 2524-32.

[29] Singaraja RR, Brunham LR, Visscher H, Kastelein JJ, Hayden MR. Efflux and atherosclerosis: the clinical and biochemical impact of variations in the ABCA1 gene. Arterioscler Thromb Vasc Biol 2003; 23: 1322-32.

[30] Clee SM, Zwinderman AH, Engert JC, *et al.* Common genetic variation in abca1 is associated with altered lipoprotein levels and a modified risk for coronary artery disease. Circulation 2001; 103: 1198-205.

[31] McNeish J, Aiello RJ, Guyot D, *et al.* High density lipoprotein deficiency and foam cell accumulation in mice with targeted disruption of ATP-binding cassette transporter-1. Proc Natl Acad Sci U S A 2000; 97: 4245-50.

[32] Aiello RJ, Brees D, Bourassa PA, *et al.* Increased atherosclerosis in hyperlipidemic mice with inactivation of ABCA1 in macrophages. Arterioscler Thromb Vasc Biol 2002; 22: 630-7.

[33] Timmins JM, Lee JY, Boudyguina E, *et al.* Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. J Clin Invest 2005; 115: 1333-42.

[34] Ragozin S, Niemeier A, Laatsch A, *et al.* Knockdown of hepatic ABCA1 by RNA interference decreases plasma HDL cholesterol levels and influences postprandial lipemia in mice. Arterioscler Thromb Vasc Biol 2005; 25: 1433-8.

[35] Brunham LR, Kruit JK, Iqbal J, *et al.* Intestinal ABCA1 directly contributes to HDL biogenesis *in vivo*. J Clin Invest 2006; 116: 1052-62.

[36] Hamilton RL, Williams MC, Fielding CJ, Havel RJ. Discoidal bilayer structure of nascent high density lipoproteins from perfused rat liver. J Clin Invest 1976; 58: 667-80.

[37] Green PH, Tall AR, Glickman RM. Rat intestine secretes discoid high density lipoprotein. J Clin Invest 1978; 61: 528-34.

[38] Brunham LR, Singaraja RR, Duong M, *et al.* Tissue-specific roles of ABCA1 influence susceptibility to atherosclerosis. Arterioscler Thromb Vasc Biol 2009; 29: 548-54.

[39] van Eck M, Bos IS, Kaminski WE, *et al.* Leukocyte ABCA1 controls susceptibility to atherosclerosis and macrophage recruitment into tissues. Proc Natl Acad Sci USA 2002; 99: 6298-303.

[40] van Eck M, Singaraja RR, Ye D, *et al.* Macrophage ATP-binding cassette transporter A1 overexpression inhibits atherosclerotic lesion progression in low-density lipoprotein receptor knockout mice. Arterioscler Thromb Vasc Biol 2006; 26: 929-34.

[41] Tearle RG, Belote JM, McKeown M, Baker BS, Howells AJ. Cloning and characterization of the scarlet gene of Drosophila melanogaster. Genetics 1989; 122: 595-606.

[42] Dreesen TD, Johnson DH, Henikoff S. The brown protein of Drosophila melanogaster is similar to the white protein and to components of active transport complexes. Mol Cell Biol 1988; 8: 5206-15.

[43] Mauldin JP, Nagelin MH, Wojcik AJ, *et al.* Reduced expression of ATP-binding cassette transporter G1 increases cholesterol accumulation in macrophages of patients with type 2 diabetes mellitus. Circulation 2008; 117: 2785-92.

[44] Schou J, Frikke-Schmidt R, Nordestgaard B, *et al.* Functional mutation in ABCG1 predicts risk of ischemic heart disease in the general population. XV International Symposium on Atherosclerosis, workshop VI-4-Genetics: Genetics of cardiovascular disease. Boston, MA, USA 2009.

[45] Furuyama S, Uehara Y, Zhang B, *et al.* Genotypic Effect of ABCG1 gene promoter -257T>G polymorphism on coronary artery disease severity in Japanese men. J Atheroscler Thromb 2009; 16: 194-200.

[46] Kennedy MA, Barrera GC, Nakamura K, *et al.* ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. Cell Metab 2005; 1: 121-31. http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B7MFH-4FGRF79-7&_user=530453&_coverDate=02%2F28%2F2005&_fmt=full&_orig=search&_cdi=23259&view=c&_acct=C000026638&_version=1&_urlVersion=0&_userid=530453&md5=13dad7981e35ac1931c465de0eff1bd2&ref=full

[47] Baldán A, Pei L, Lee R, *et al.* Impaired development of atherosclerosis in hyperlipidemic Ldlr$^{-/-}$ and ApoE$^{-/-}$ mice transplanted with Abcg1$^{-/-}$ bone marrow. Arterioscler Thromb Vasc Biol 2006; 26: 2301-7.

[48] Lammers B, Out R, Hildebrand RB, *et al.* Independent protective roles for macrophage Abcg1 and Apoe in the atherosclerotic lesion development. Atherosclerosis 2009; 205: 420-6.

[49] Ranalletta M, Wang N, Han S, Yvan-Charvet L, Welch C, Tall AR. Decreased atherosclerosis in low-density lipoprotein receptor knockout mice transplanted with Abcg1−/− bone marrow. Arterioscler Thromb Vasc Biol 2006; 26: 2308-15.

[50] Out R, Hoekstra M, Hildebrand RB, *et al.* Macrophage ABCG1 deletion disrupts lipid homeostasis in alveolar macrophages and moderately influences atherosclerotic lesion development in LDL receptor-deficient mice. Arterioscler Thromb Vasc Biol 2006; 26: 2295-300.

[51] Chan B, Wilkinson A, Fan J, *et al.* Overexpression of human ABCG1 does not affect atherosclerosis in fat-fed ApoE-deficient mice. Arterioscler Thromb Vasc Biol 2008; 28: 1731-7.

[52] Basso F, Amar MJ, Wagner EM, *et al.* Enhanced ABCG1 expression increases atherosclerosis in LDLr-KO mice on a western diet. Biochem Biophys Res Commun 2006; 351: 398-404.

[53] Gelissen IC, Harris M, Rye KA, *et al.* ABCA1 and ABCG1 synergize to mediate cholesterol export to apoA-I. Arterioscler Thromb Vasc Biol 2006; 26: 534-40.

[54] Out R, Jessup W, Le Goff W, *et al.* Coexistence of foam cells and hypocholesterolemia in mice lacking the ABC transporters A1 and G1. Circ Res 2008; 102: 113-20.

[55] Yvan-Charvet L, Ranalletta M, Wang N, *et al.* Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice. J Clin Invest 2007; 117: 3900-8.

[56] Wang X, Collins HL, Ranalletta M, *et al.* Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport *in vivo*. J Clin Invest 2007; 117: 2216-24.

[57] Out R, Hoekstra M, Habets K, *et al.* Combined deletion of macrophage ABCA1 and ABCG1 leads to massive lipid accumulation in tissue macrophages and distinct atherosclerosis at relatively low plasma cholesterol levels. Arterioscler Thromb Vasc Biol 2008; 28: 258-64.

[58] Harris RL, Musher DM, Bloom K, *et al.* Manifestation of sepsis. Arch Intern Med 1987; 1477: 1895-906.

[59] Dobrovolskaia MA, Vogel SN. Toll receptors, CD14, and macrophage activation and deactivation by LPS. Microbes Infect 2002; 4: 903-14.

[60] Francone OL, Royer L, Boucher G, *et al.* Increased cholesterol deposition, expression of scavenger receptors, and response to chemotactic factors in Abca1-deficient macrophages. Arterioscler Thromb Vasc Biol 2005; 25: 1198-205.

[61] O'Connell BJ, Denis M, Genest J. Cellular physiology of cholesterol efflux in vascular endothelial cells. Circulation 2004; 110: 2881-8.

[62] Berliner JA, Navab M, Fogelman AM, *et al.* Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. Circulation 1995; 91: 2488-96.

[63] Berliner JA, Vora DK, Shih PT. Control of leukocyte adhesion and activation in atherogenesis. Vascular Adhesion and Inflammation 2001; 239-56.

[64] Terasaka N, Yu S, Yvan-Charvet L, *et al.* ABCG1 and HDL protect against endothelial dysfunction in mice fed a high-cholesterol diet. J Clin Invest 2008; 118: 3701-13.

[65] Barter PJ, Nicholls S, Rye KA, Anantharamaiah GM, Navab M, Fogelman AM. Antiinflammatory properties of HDL. Circ Res 2004; 95: 764-72.

[66] Navab M, Hama SY, Anantharamaiah GM, *et al.* Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: steps 2 and 3. J Lipid Res 2000; 41: 1495-508.

[67] Aiello RJ, Brees D, Francone OL. ABCA1-deficient mice: insights into the role of monocyte lipid efflux in HDL formation and inflammation. Arterioscler Thromb Vasc Biol 2003; 23: 972-80.

[68] Zhu X, Lee JY, Timmins JM, *et al.* Increased cellular free cholesterol in macrophage-specific Abca1 knock-out mice enhances pro-inflammatory response of macrophages. J Biol Chem 2008; 283: 22930-41.

[69] Koseki M, Hirano K, Masuda D, *et al.* Increased lipid rafts and accelerated lipopolysaccharide-induced tumor necrosis factor-alpha secretion in Abca1-deficient macrophages. J Lipid Res 2007; 48: 299-306.

[70] Sun Y, Ishibashi M, Seimon T, *et al.* Free cholesterol accumulation in macrophage membranes activates Toll-like receptors and p38 mitogen-activated protein kinase and induces cathepsin K. Circ Res 2009; 104: 455-65.

[71] Tang C, Vaughan AM, Anantharamaiah GM, Oram JF. Janus kinase 2 modulates the lipid-removing but not protein-stabilizing interactions of amphipathic helices with ABCA1. J Lipid Res 2006; 47: 107-14.

[72] Tang C, Vaughan AM, Oram JF. Janus kinase 2 modulates the apolipoprotein interactions with ABCA1 required for removing cellular cholesterol. J Biol Chem 2004; 279: 7622-8.

[73] Aaronson DS, Horvath CM. A road map for those who don't know JAK-STAT. Science 2002; 296: 1653-5.

[74] Williams LM, Sarma U, Willets K, Smallie T, Brennan F, Foxwell BM. Expression of constitutively active STAT3 can replicate the cytokine-suppressive activity of interleukin-10 in human primary macrophages. J Biol Chem 2007; 282: 6965-75.

[75] Tang C, Liu Y, Kessler PS, Vaughan AM, Oram JF. The macrophage cholesterol exporter ABCA1 functions as an anti-inflammatory receptor. J Biol Chem 2009; 284: 32336-43.

[76] Wojcik AJ, Skaflen MD, Srinivasan S, Hedrick CC. A critical role for ABCG1 in macrophage inflammation and lung homeostasis. J Immunol 2008; 180: 4273-82.

[77] Schmitz G, Orsó E. CD14 signalling in lipid rafts: new ligands and co-receptors. Curr Opin Lipidol 2002; 13: 513-521.

[78] Yvan-Charvet L, Welch C, Pagler TA, *et al.* Increased inflammatory gene expression in ABC transporter-deficient

macrophages: free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions. Circulation 2008; 118: 1837-47.

[79] Yvan-Charvet L, Wang N, Tall AR. Role of HDL, ABCA1, and ABCG1 transporters in cholesterol efflux and immune responses. Arterioscler Thromb Vasc Biol 2010; 30: 139-43.

[80] Sano O, Kobayashi A, Nagao K, *et al.* Sphingomyelin-dependence of cholesterol efflux mediated by ABCG1. J Lipid Res 2007; 48: 2377-84.

[81] Maxfield FR, Tabas I. Role of cholesterol and lipid organization in disease. Nature 2005; 438: 612-21.

[82] Larsson DA, Baird S, Nyhalah JD, Yuan XM, Li W. Oxysterol mixtures, in atheroma-relevant proportions, display synergistic and proapoptotic effects. Free Radic Biol Med 2006; 41: 902-10.

[83] Lusis AJ. Atherosclerosis. Nature 2000; 407: 233-4.

[84] Terasaka N, Wang N, Yvan-Charvet L, Tall AR. High-density lipoprotein protects macrophages from oxidized low-density lipoprotein-induced apoptosis by promoting efflux of 7-ketocholesterol via ABCG1. Proc Natl Acad Sci USA 2007; 104: 15093-8.

[85] Engel T, Kannenberg F, Fobker M, *et al.* Expression of ATP binding cassette-transporter ABCG1 prevents cell death by transporting cytotoxic 7β-hydroxycholesterol. FEBS Lett 2007; 581: 1673-80.

[86] Whetzel AM, Sturek JM, Nagelin MH, *et al.* ABCG1 deficiency in mice promotes endothelial activation and monocyte-endothelial interactions. Arterioscler Thromb Vasc Biol 2010; 30: 809-17.

[87] Bungert S, Molday LL, Molday RS. Membrane topology of the ATP binding cassette transporter ABCR and its relationship to ABC1 and related ABCA transporters: identification of N-linked glycosylation sites. J Biol Chem 2001; 276: 23539-46.

[88] Fitzgerald ML, Mendez AJ, Moore KJ, Andersson LP, Panjeton HA, Freeman MW. ATP-binding cassette transporter A1 contains an NH2-terminal signal anchor sequence that translocates the protein's first hydrophilic domain to the exoplasmic space. J Biol Chem 2001; 276: 15137-45.

[89] Langmann T, Klucken J, Reil M, *et al.* Molecular cloning of the human ATP-binding cassette transporter 1 (hABC1): evidence for sterol-dependent regulation in macrophages. Biochem Biophys Res Commun 1999; 257: 29-33.

[90] Lawn RM, Wade DP, Couse TL, Wilcox JN. Localization of human ATP-binding cassette transporter 1 (ABC1) in normal and atherosclerotic tissues. Arterioscler Thromb Vasc Biol 2001; 21: 378-85.

[91] Wellington CL, Walker EK, Suarez A, *et al.* ABCA1 mRNA and protein distribution patterns predict multiple different roles and levels of regulation. Lab Invest 2002; 82: 273-83.

[92] Wang N, Silver DL, Thiele C, Tall AR. ATP-binding cassette transporter A1 (ABCA1) functions as a cholesterol efflux regulatory protein. J Biol Chem 2001; 276: 23742-7.

[93] Wang N, Silver DL, Costet P, Tall AR. Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. J Biol Chem 2000; 275: 33053-8.

[94] Hamon Y, Broccardo C, Chambenoit O, *et al.* ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. Nat Cell Biol 2000; 2: 399-406.

[95] Fitzgerald ML, Mendez AJ, Moore KJ, Andersson LP, Panjeton HA, Freeman MW. ATP-binding cassette transporter A1 contains an NH2-terminal signal anchor sequence that translocates the protein's first hydrophilic domain to the exoplasmic space. J Biol Chem 2001; 276: 15137-45.

[96] Orso E, Broccardo C, Kaminski WE, *et al.* Transport of lipids from Golgi to plasma membrane is defective in tangier disease patients and Abc1-deficient mice. Nat Genet 2000; 24: 192-6.

[97] Oram JF, Heinecke JW. ATP-binding cassette transporter A1: a cell cholesterol exporter that protects against cardiovascular disease. Physiol Rev 2005; 85: 1343-72.

[98] Fitzgerald ML, Morris AL, Rhee JS, Andersson LP, Mendez AJ, Freeman MW. Naturally occurring mutations in the largest extracellular loops of ABCA1 can disrupt its direct interaction with apolipoprotein A-I. J Biol Chem 2002; 277: 33178-87.

[99] Denis M, Haidar B, Marcil M, Bouvier M, Krimbou L, Genest J Jr. Molecular and cellular physiology of apolipoprotein A-I lipidation by the ATP-binding cassette transporter A1 (ABCA1). J Biol Chem 2004; 279: 7384-94.

[100] Vaughan AM, Oram JF. ABCA1 redistributes membrane cholesterol independent of apolipoprotein interactions. J Lipid Res 2003; 44: 1373-80.

[101] Yancey PG, Bortnick AE, Kellner-Weibel G, de la Llera-Moya M, Phillips MC, Rothblat GH. Importance of different pathways of cellular cholesterol efflux. Arterioscler Thromb Vasc Biol 2003; 23: 712-9.

[102] Rye KA, Barter PJ. Formation and metabolism of prebeta-migrating, lipid-poor apolipoprotein A-I. Arterioscler Thromb Vasc Biol 2004; 24: 421-8.

[103] Yokoyama S. Assembly of high-density lipoprotein. Arterioscler Thromb Vasc Biol 2006; 26: 20-7.

[104] Liadaki KN, Liu T, Xu S, *et al.* Binding of high density lipoprotein (HDL) and discoidal reconstituted HDL to the HDL receptor scavenger receptor class B type I. Effect of lipid association and APOA-I mutations on receptor binding. J Biol Chem 2000; 275: 21262-71.

[105] Oram JF. ATP-binding cassette transporter A1 and cholesterol trafficking. Curr Opin Lipidol 2002; 13: 373-81.

[106] Santamarina-Fojo S, Remaley AT, Neufeld EB, Brewer HB Jr. Regulation and intracellular trafficking of the ABCA1 transporter. J Lipid Res 2001; 42: 1339-45.

[107] Takahashi Y, Smith JD. Cholesterol efflux to apolipoprotein AI involves endocytosis and resecretion in a calcium-dependent pathway. Proc Natl Acad Sci USA. 1999; 96:11358-11363.

[108] Smith JD, Waelde C, Horwitz A, Zheng P. Evaluation of the role of phosphatidylserine translocase activity in ABCA1-mediated lipid efflux. *J Biol Chem.* 2002; 277: 17797-803.

[109] Hassan HH, Bailey D, Lee DY, *et al.* Quantitative analysis of ABCA1-dependent compartmentalization and trafficking of apolipoprotein A-I: implications for determining cellular kinetics of nascent high density lipoprotein biogenesis. J Biol Chem 2008; 283: 11164-75.

[110] Chen W, Wang N, Tall AR. A PEST deletion mutant of ABCA1 shows impaired internalization and defective cholesterol efflux from late endosomes. J Biol Chem 2005; 280: 29277-81.

[111] Costet P, Luo Y, Wang N, Tall AR. Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. J Biol Chem 2000; 275: 28240-5.

[112] Schwartz K, Lawn RM, Wade DP. ABC1 gene expression and ApoA-I-mediated cholesterol efflux are regulated by LXR. Biochem Biophys Res Commun 2000; 274: 794-802.

[113] Schmitz G, Buechler C. ABCA1: regulation, trafficking and association with heteromeric proteins. Ann Med 2002; 34: 334-47.

[114] Oram JF, Lawn RM, Garvin MR, Wade DP. ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. J Biol Chem 2000; 275: 34508-11.

[115] Wang N, Chen W, Linsel-Nitschke P, *et al.* A PEST sequence in ABCA1 regulates degradation by calpain protease and stabilization of ABCA1 by apoA-I. J Clin Invest 2003; 111: 99-107.

[116] Wang Y, Oram JF. Unsaturated fatty acids inhibit cholesterol efflux from macrophages by increasing degradation of ATP-binding cassette transporter A1. J Biol Chem 2002; 277: 5692-7.

[117] Martinez LO, Agerholm-Larsen B, Wang N, Chen W, Tall AR. Phosphorylation of a pest sequence in ABCA1 promotes calpain degradation and is reversed by ApoA-I. J Biol Chem 2003; 278: 37368-74.

[118] See RH, Caday-Malcolm RA, Singaraja RR, *et al.* Protein kinase A site-specific phosphorylation regulates ATP-binding cassette A1 (ABCA1)-mediated phospholipid efflux. J Biol Chem 2002; 277: 41835-42.

[119] Haidar B, Denis M, Krimbou L, Marcil M, Genest J Jr. cAMP induces ABCA1 phosphorylation activity and promotes cholesterol efflux from fibroblasts. J Lipid Res 2002; 43: 2087-94.

[120] Yamauchi Y, Hayashi M, AbeDohmae S, Yokoyama S. Apolipoprotein A-I activates protein kinase C alpha signaling to phosphorylate and stabilize ATP binding cassette transporter A1 for the high density lipoprotein assembly. J Biol Chem 2003; 278: 47890-7.

[121] Arakawa R, Hayashi M, Remaley AT, Brewer BH, Yamauchi Y, Yokoyama S. Phosphorylation and stabilization of ATP binding cassette transporter A1 by synthetic amphiphilic helical peptides. J Biol Chem 2004; 279: 6217-20.

[122] Roosbeek S, Peelman F, Verhee A, *et al.* Phosphorylation by protein kinase CK2 modulates the activity of the ATP binding cassette A1 transporter. J Biol Chem 2004; 279: 37779-88.

[123] Arakawa R, Yokoyama S. Helical apolipoproteins stabilize ATP-binding cassette transporter A1 by protecting it from thiol protease-mediated degradation. J Biol Chem 2002; 277: 22426-9.

[124] Feng B, Tabas I. ABCA1-mediated cholesterol efflux is defective in free cholesterol-loaded macrophages. Mechanism involves enhanced ABCA1 degradation in a process requiring full NPC1 activity. J Biol Chem 2002; 277: 43271-80.

[125] Wang Y, Kurdi-Haidar B, Oram JF. LXR-mediated activation of macrophage stearoyl-CoA desaturase generates unsaturated fatty acids that destabilize ABCA1. J Lipid Res 2004; 45: 972-80.

[126] Walker JE, Saraste M, Runswick MJ, Gay NJ. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J 1982; 1: 945-51.

[127] Savary S, Denizot F, Luciani M, Mattei M, Chimini G. Molecular cloning of a mammalian ABC transporter homologous to Drosophila white gene. Mamm Genome 1996; 7: 673-6.

[128] Croop JM, Tiller GE, Fletcher JA, *et al.* Isolation and characterization of a mammalian homolog of the Drosophila white gene. Gene 1997; 185: 77-85.

[129] Chen H, Rossier C, Lalioti MD, *et al.* Cloning of the cDNA for a human homologue of the Drosophila white gene and mapping to chromosome 21q22.3. Am J Hum Genet 1996; 59: 66-75.

[130] Kennedy MA, Venkateswaran A, Tarr PT, *et al.* Characterization of the human abcg1 gene. Liver x receptor activates an internal promoter that produces a novel transcript encoding an alternative form of the protein. J Biol Chem 2001; 276: 39438-47.

[131] Lorkowski S, Rust S, Engel T, *et al.* Genomic sequence and structure of the human ABCG1 (ABC8) gene. Biochem Biophys Res Commun 2001; 280: 121-31.

[132] Nakamura K, Kennedy MA, Baldán A, Bojanic DD, Lyons K, Edwards PA. Expression and regulation of multiple murine ATP-binding cassette transporter G1 mRNAs/isoforms that stimulate cellular cholesterol efflux to high density lipoprotein. J Biol Chem 2004; 279: 45980-9.

[133] Schmitz G, Langmann T, Heimerl S. Role of ABCG1 and other ABCG family members in lipid metabolism. J Lipid Res 2001; 42: 1513-20.

[134] Engel T, Bode G, Lueken A, *et al.* Expression and functional characterization of ABCG1 splice variant ABCG1. FEBS Lett 2006; 580: 4551-9.

[135] Gelissen IC, Cartland S, Brown AJ, *et al.* Expression and stability of two isoforms of ABCG1 in human vascular cells. Atherosclerosis 2010; 208: 75-82.

[136] Smith JD. Insight into ABCG1-mediated cholesterol efflux. Arterioscler Thromb Vasc Biol 2006; 26: 1198-200.

[137] Vaughan AM, Oram JF. ABCG1 redistributes cell cholesterol to domains removable by high density lipoprotein but not by lipid-depleted apolipoproteins. J Biol Chem 2005; 280: 30150-7.

[138] Small DM. Role of ABC transporters in secretion of cholesterol from liver into bile. Proc Natl Acad Sci USA 2003; 100: 4-6.

[139] Kobayashi A, Takanezawa Y, Hirata T, *et al.* Efflux of sphingomyelin, cholesterol, and phosphatidylcholine by ABCG1. J Lipid Res 2006; 47: 1791-802.

[140] Wang N, Ranalletta M, Matsuura F, Peng F, Tall AR. LXR-induced redistribution of ABCG1 to plasma membrane in macrophages enhances cholesterol mass efflux to HDL. Arterioscler Thromb Vasc Biol 2006; 26: 1310-6.

[141] Larrede S, Quinn CM, Jessup W, *et al.* Stimulation of cholesterol efflux by LXR agonists in cholesterol-loaded human macrophages is ABCA1-dependent but ABCG1-independent. Arterioscler Thromb Vasc Biol 2009; 29: 1930-6.

[142] Chawla A, Boisvert WA, Lee CH, *et al.* A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. Mol Cell 2001; 7: 161-71.

[143] Akiyama TE, Sakai S, Lambert G, *et al.* Conditional disruption of the peroxisome proliferator-activated receptor gamma gene in mice results in lowered expression of ABCA1, ABCG1, and apoE in macrophages and reduced cholesterol efflux. Mol Cell Biol 2002; 22: 2607-19.

[144] Li AC, Binder CJ, Gutierrez A, *et al.* Differential inhibition of macrophage foam-cell formation and atherosclerosis in mice by PPARalpha, beta/delta and gamma. J Clin Invest 2004; 114: 1564-76.

[145] Uehara Y, Miura S, von Eckardstein A, *et al.* Unsaturated fatty acids suppress the expression of the ATP-binding cassette transporter G1 (ABCG1) and ABCA1 genes via an LXR/RXR responsive element. Atherosclerosis 2007; 191: 11-21.

[146] Yamamoto SY, Takahashi Y, Hada T, *et al.* Mammalian arachidonate 12-lipoxygenases. Adv Exp Med Biol 1997; 400: 127-31.

[147] Nagelin MH, Srinivasan S, Nadler JL, Hedrick CC. 12/15-lipoxygenase activity increases the degradation of macrophage ATP-binding cassette transporter G1. Arterioscler Thromb Vasc Biol 2008; 28: 1811-9.

[148] Nagelin MH, Srinivasan S, Nadler JL, Hedrick CC. Murine 12/15-lipoxygenase regulates ATP-binding cassette transporter G1 protein degradation through p38- and JNK2-dependent pathways. J Biol Chem 2009; 284: 31303-14.

[149] Mauldin JP, Srinivasan S, Mulya A, *et al.* Reduction in ABCG1 in Type 2 diabetic mice increases macrophage foam cell formation. J Biol Chem 2006; 281: 21216-24.

[150] Zhou H, Tan KC, Shiu SW, Wong Y. Determinants of leukocyte adenosine triphosphate-binding cassette transporter G1 gene expression in type 2 diabetes mellitus. Metabolism 2008; 57: 1135-40.

[151] Passarelli M, Tang C, McDonald TO, *et al.* Advanced glycation end product precursors impair ABCA1-dependent cholesterol removal from cells. Diabetes 2005; 54: 2198-205.

[152] Laffitte BA, Repa JJ, Joseph SB, *et al.* LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. Proc Natl Acad Sci USA 2001; 98: 507-12.

[153] Repa JJ, Berge KE, Pomajzl C, Richardson JA, Hobbs H, Mangelsdorf DJ. Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. J Biol Chem 2002; 277: 18793-800.

[154] Tall AR. Plasma cholesteryl ester transfer protein. J Lipid Res 1993; 34: 1255-74.

[155] Lusa S, Jauhiainen M, Metso J, Somerharju P, Ehnholm C. The mechanism of human plasma phospholipid transfer protein-induced enlargement of high-density lipoprotein particles: evidence for particle fusion. Biochem J 1996; 313: 275-82.

[156] Joseph SB, Bradley MN, Castrillo A. LXR-dependent gene expression is important for macrophage survival and the innate immune response. Cell 2004; 119: 299-309.

[157] Valledor AF, Hsu LC, Ogawa S, Sawka-Verhelle D, Karin M, Glass CK. Activation of liver X receptors and retinoid X receptors prevents bacterial-induced macrophage apoptosis. Proc Natl Acad Sci USA 2004; 101: 17813-8.

[158] Joseph SB, McKilligin E, Pei L, *et al.* Synthetic LXR ligand inhibits the development of atherosclerosis in mice. Proc Natl Acad Sci USA 2002; 99: 7604-9.

[159] Miao B, Zondlo S, Gibbs S, *et al.* Raising HDL cholesterol without inducing hepatic steatosis and hypertriglyceridemia by a selective LXR modulator. J Lipid Res 2004; 45: 1410-7.

[160] Tontonoz P, Mangelsdorf DJ. Liver x receptor signaling pathways in cardiovascular disease. Mol Endocrinol 2003; 17: 985-93.

[161] Levin N, Bisschoff ED, Daige CL. Macrophage liver X receptor is required for antiatherogenic activity of LXR agonists. Arterioscler Thromb Vasc Biol 2005; 25: 135-42.

[162] Verschuren L, de Vries-van der Weij J, Zadelaar S, Kleemann R, Kooistra T. LXR agonist suppresses atherosclerotic lesion growth and promotes lesion regression in apoE*3Leiden mice: time course and mechanisms. J Lipid Res 2009; 50: 301-11.

[163] Schultz JR, Tu H, Luk A, *et al.* Role of LXRs in control of lipogenesis. Genes Dev 2000; 14: 2831-8.

[164] Grefhorst A, Elzinga BM, Voshol PJ, *et al.* Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. J Biol Chem 2002; 277: 34182-90.

[165] Repa JJ, Liang G, Ou J, *et al.* Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. Genes Dev 2000; 14: 2819-30.

[166] Repa JJ, Turley SD, Lobaccaro JA, *et al.* Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. Science 2000; 289: 1524-9.

[167] Lund EG, Peterson LB, Adams AD, *et al.* Different roles of liver X receptor alpha and beta in lipid metabolism: effects of an alpha-selective and a dual agonist in mice deficient in each subtype. Biochem Pharmacol 2006; 71: 453-63.

[168] Quinet EM, Savio DA, Halpern AR, *et al.* Liver X receptor (LXR)-beta regulation in LXRalpha-deficient mice: implications for therapeutic targeting. Mol Pharmacol 2006; 70: 1340-9.

[169] Williams S, Bledsoe RK, Collins JL, *et al.* X-ray crystal structure of the liver X receptor beta ligand binding domain: Regulation by a histidine-tryptophan switch. J Biol Chem 2003; 278: 27138-43.

[170] Svensson S, Ostberg T, Jacobsson M, *et al.* Crystal structure of the heterodimeric complex of LXRalpha and RXRbeta ligand-binding domains in a fully agonistic conformation. EMBO J 2003; 22: 4625-33.

[171] Adams CM, Reitz J, De Brabander JK, *et al.* Cholesterol and 25-hydroxycholesterol inhibit activation of SREBPs by different mechanisms, both involving SCAP and Insigs. J Biol Chem 2004; 279: 52772-80.

[172] Beyea MM, Heslop CL, Sawyez CG, *et al.* Selective up-regulation of LXR-regulated genes ABCA1, ABCG1, and APOE in macrophages through increased endogenous synthesis of 24(S), 25-epoxycholesterol. J Biol Chem 2007; 282: 5207-16.

[173] Peng D, Hiipakka RA, Dai Q, *et al.* Antiatherosclerotic effects of a novel synthetic tissue-selective steroidal liver X receptor agonist in low-density lipoprotein receptor-deficient mice. J Pharmacol Exp Ther 2008; 327: 332-42.

[174] Navab M, Anantharamaiah GM, Hama S, *et al.* Oral administration of an Apo A-I mimetic peptide synthesized from D-amino acids dramatically reduces atherosclerosis in mice independent of plasma cholesterol. Circulation 2002; 105: 290-2.

[175] Navab M, Anantharamaiah GM, Reddy ST, *et al.* Oral D-4F causes formation of pre-beta high-density lipoprotein and improves high density lipoprotein-mediated cholesterol efflux and reverse cholesterol transport from macrophages in apolipoprotein E-null mice. Circulation 2004; 109: 3215-20.

[176] Zheng L, Nukuna B, Brennan ML, *et al.* Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. J Clin Invest 2004; 114: 529-41.

[177] Remaley AT, Thomas F, Stonik JA, *et al.* Synthetic amphipathic helical peptides promote lipid efflux from cells by an ABCA1-dependent and an ABCA1-independent pathway. J Lipid Res 2003; 44: 828-36.

[178] Sethi AA, Stonik JA, Thomas F, *et al.* Asymmetry in the lipid affinity of bihelical amphipathic peptides. A structural determinant for the specificity of ABCA1-dependent cholesterol efflux by peptides. J Biol Chem 2008; 283: 32273-82.

[179] Kunitake ST, La Sala KJ, Kane JP. Apolipoprotein A-I-containing lipoproteins with pre-beta electrophoretic mobility. J Lipid Res 1985; 26: 549-55.

[180] Asztalos BF, Schaefer EJ, Horvath KV, *et al.* Role of LCAT in HDL remodeling: investigation of LCAT deficiency states. J Lipid Res 2007; 48: 592-9.

[181] Shah PK, Yano J, Reyes O, *et al.* High-dose recombinant apolipoprotein A-I (milano) mobilizes tissue cholesterol and rapidly reduces plaque lipid and macrophage content in apolipoprotein e-deficient mice: potential implications for acute plaque stabilization. Circulation 2001; 103: 3047-50.

[182] Nissen SE, Tsunoda T, Tuzcu EM, *et al.* Effect of recombinant ApoA-IMilano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. JAMA 2003; 290: 2292-300.

[183] Navab M, Anantharamaiah GM, Reddy ST, Fogelman AM. Apolipoprotein A-I mimetic peptides and their role in atherosclerosis prevention. Nat Clin Pract Cardiovasc Med 2006; 3: 540-7.

[184] Sacks FM, Rudel LL, Conner A, *et al.* Selective delipidation of plasma HDL enhances reverse cholesterol transport *in vivo*. J Lipid Res 2009; 50: 894-907.

Received: February 11, 2010 Revised: April 06, 2010 Accepted: April 06, 2010

The Canalicular Bile Salt Export Pump BSEP (ABCB11) as a Potential Therapeutic Target

Bruno Stieger[*,1] and Ulrich Beuers[2]

[1]*Division of Clinical Pharmacology and Toxicology, University Hospital, Zurich, Switzerland*

[2]*Department of Gastroenterology and Hepatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands*

Abstract: Bile formation is a key function of the liver and is driven by active secretion of bile salts and other organic compounds into the biliary tree. Bile salts represent the major organic constituent of bile. They are released with bile into the small intestine, where they are almost quantitatively reabsorbed and transported via the portal circulation back to the liver. In the liver, they are taken up into hepatocytes and secreted into bile. This cycling between the liver and the small intestine is called enterohepatic circulation of bile salts. Bile salts are secreted from hepatocytes into the bile by the bile salt export pump BSEP. This step constitutes the rate-limiting step of handling of bile salts in the liver and is the major driving force of the enterohepatic circulation of bile salts. Improper functioning of BSEP leads to an accumulation of bile salts within hepatocytes, where bile salts become cytotoxic. If persistent, accumulation of bile salts in hepatocytes will lead to liver disease. This review summarizes the essential concepts of bile formation and the current knowledge of mechanisms known to impair BSEP function. Finally, it sets the current therapeutic approaches for cholestatic liver disease into perspective to the pathophysiologic mechanisms of impaired BSEP function.

Keywords: ABC transporter, bile formation, BSEP, cholestasis, drug, liver injury.

PHYSIOLOGY OF BILE FORMATION

The liver is strategically located between the gut and the systemic circulation. Nutrients as well as drugs and other xenobiotics, once absorbed by the gut pass via the portal blood to the liver and from there, if not retained by the liver, to the systemic circulation. In the small intestine, digestion and absorption of lipids requires bile salts. Bile salts represent two thirds of organic constituents of bile. Bile is produced by the liver, whereby hepatocytes form about 60 to 75 % and biliary epithelia about 25 to 40 % of bile fluid in humans. Bile is collected in the gall bladder in between meals and released from the gall bladder into the duodenum after a meal. Important constituents of bile, in addition to bile salts, are lipids (phosphatidylcholine and cholesterol), organic anions (bilirubin and others) and small ions [1]. Hepatic bile flow is composed of bile salt-dependent bile flow, which is driven by bile salt secretion and bile salt-independent bile flow, which is mainly driven by secretion of organic anions [2].

Bile salts are a metabolic product of cholesterol and are synthesized in a multistep process in hepatocytes [3]. After synthesis, they are rapidly conjugated by linking their carboxylic acid group to the amino group of taurine or glycine forming an amide [4] and secreted into the canaliculi for delivery into the biliary tree [5, 6]. In the small intestine, bile salts promote the digestion of fat and absorption of lipids and fat soluble vitamins [5, 7]. Bile salts are absorbed to more than 90 % in the small intestine and cycle back to the liver via the portal circulation. In the liver, bile salts are taken up from the sinusoidal blood plasma into hepatocytes for secretion into the biliary tree [8]. This cycling between the gut and the liver is termed enterohepatic circulation of bile salts and a "motus circularis bilis" has first been described in 1690 by Mauritius Reverhorst and in 1710 by Giovanni Alfonso Borelli [9]. For efficient transport of bile salts from the sinusoids into the canaliculi as well as for controlling this process, hepatocytes are equipped with an elaborate array of transporters and regulatory mechanisms [10-12]. Hence, hepatocytes are the principle cells involved in bile formation and provide the driving force for the enterohepatic circulation of bile salts. A tight control of bile salt concentration within hepatocytes is essential, as they are amphipathic molecules and display cytotoxic and at high concentrations even detergent properties [7]. Hence, any surplus of bile salts within hepatocytes can become damaging or even lethal to the cells [13, 14].

At the basolateral plasma membrane of hepatocytes, bile salts are mostly taken up in a sodium-dependent manner and to a minor extent via sodium-independent processes. Sodium-dependent uptake of bile salts is mediated by the sodium-taurocholate cotransporting polypeptide NTCP (*SLC10A1*), which shows a preference for conjugated bile salts [8, 15], while sodium-independent uptake of bile salts shows a preference for unconjugated bile acids and is fostered by organic anion transporting polypeptides or OATPs (*SLCOs*), namely OATP1B1 and OATP1B3 [10, 11]. As an additional OATP, OATP2B1 is also expressed in hepatocytes but does not mediate transport of conjugated bile salts [10, 16]. Importantly, OATPs are also key mediators of hepatocellular drug and xenobiotic uptake, whereby OATP1B1 and OATP1B3 exhibit overlapping substrate specificities [17]. Knowledge about intracellular transport of bile salts from the basolateral cell pole across hepatocytes to the canalicular plasma membrane is limited, but bile salt binding proteins are almost certainly involved [18, 19].

*Address correspondence to this author at the University Hospital, Division of Clinical Pharmacology and Toxicology, 8091 Zürich, Switzerland; Tel: +41-44-255-2068; Fax: +41-44-255-4411;
E-mail: bstieger@kpt.unizh.ch

1389-4501/11 $58.00+.00 © 2011 Bentham Science Publishers Ltd.

Canalicular export of bile salts occurs against a steep concentration gradient and is mediated by the bile salt export pump BSEP (ABCB11), which is a member of the ATP-binding cassette (ABC) transporter super family [8, 20, 21]. The rate limiting step in the overall transport from the portal blood into bile is located at the canalicular plasma membrane [10, 22]. Hence, undisturbed functioning and proper regulation of BSEP is essential for keeping the potentially cytotoxic bile salts at a low intracellular level in hepatocytes. In rat liver exposed to a physiologic load of bile salts, the concentration of their monomeric form (i.e. soluble in the cytoplasm in their anionic form [23]) in the cytoplasm is probably < 1 μM [24]. Based on comparable total liver tissue bile salt concentrations [25], a similar value can be assumed for human hepatocytes. In the canaliculus, formation of primary bile is an isoosmotic process. Water follows the secreted solutes using a paracellular route across tight junctions and a transcellular route mediated by aquaporins, which display a distinct expression pattern in the basolateral and canalicular membrane of hepatocytes [26]. Consequently, bile salts are a crucial driving force for the generation of canalicular bile flow. Any reduction of bile flow represents a pathophysiologic situation and is called cholestasis.

Even though NTCP is predominantly a bile salt transporter it handles in addition sulfated compounds such as bromosulfophthalein and sulfated steroid metabolites [8, 15, 27, 28]. Of note, human NTCP but not rat Ntcp transports the statin rosuvastatin [29]. Experiments with isolated human hepatocytes suggest that NTCP mediates 35 % of total rosuvastatin, the remaining part being OATP-mediated. Taken together, NTCP acts not only as the key hepatocellular bile salt uptake system, but may also contribute to hepatocellular handling of steroid metabolites and some drugs. NTCP is under tight transcriptional and post-transcriptional regulation to keep intracellular bile salts below cytotoxic levels. Transcriptionally, NTCP is repressed by upregulation of the small heterodimer partner 1 (SHP-1, NR0B2), which is under direct positive control of the bile salt sensor farnesoid X receptor (FXR, NR1H4) [30]. This mechanism is species-dependent. Post-transcriptionally, NTCP exists in different phosphorylation states, which in turn regulate its recycling between an endosomal compartment and the basolateral plasma membrane. Increasing cAMP induces dephosphorylation of NTCP in the endosomal compartment from where dephosphorylated NTCP is shipped to the basolateral domain of hepatocytes [31].

OATPs have a very broad substrate pattern and transport a considerable number of endogenous substrates, metabolic end products as well as xenobiotics including drugs and toxins. Examples of endogenous substrates are bile salts; examples of metabolic end products are estrone-3-sulfate and thyroxin sulfates [16, 17, 32]. Hepatic OATPs are also subject to regulation. The transcription of OATP1B3 is under direct and the transcription of OATP1B1 under indirect control of the bile salt sensor FXR. In this context, transcription of OATP1B1 is diminished by a SHP-1 induced repression of hepatocyte nuclear factor 1α (HNF1α), the major activator of the OATP1B1 promotor [30]. Rat Oatp1a1 and Oatp1a4 have been shown to undergo phosphorylation in heterologous expression systems resulting in their functional down regulation [33, 34].

BSEP has a narrow, but species-dependent substrate specificity and transports monoanionic, conjugated bile salts [20, 21]. Human BSEP, but not rat Bsep transports the bile salt metabolite taurolithocholate-3-sulfate [35, 36]. Rat Bsep transports practically no unconjugated bile salts [37], which *in vivo* is mirrored by the clinical observation that patients with defective bile salt conjugation have very little unconjugated bile salts in their bile [38]. In addition to bile salts, human BSEP and rat Bsep are able to mediate transport of pravastatin, but not other statins [39].

The transcription of BSEP is under strong positive control by the bile salt sensor FXR [21, 30]. In addition to ligand binding for its activation, FXR requires interaction with its partner retinoid X receptor (RXR, NR2B1) to form a heterodimeric complex [40]. This complex binds to the sequence element AGGTCA, which forms an inverted repeat separated by one nucleotide (IR-1 element) on FXR responsive genes [30, 40], a process which is under additional control of coactivators and corepressors [41]. FXR is more strongly activated by hydrophobic than by hydrophilic bile salts [30, 42]. Additional FXR agonists include polyunsaturated fatty acids, hydroxylated bile alcohols, oxysterols, andosterone, dietary component from plants such as stigmasterol, cafestol [30, 43] or procyanidines found in grapes, red wine, apples and chocolate [44]. Known FXR antagonists are guggulsterone, lithocholate and the synthetic compound AGN34 [42]. Due to its central role in bile salt and hence lipid and energy homeostasis, several synthetic FXR agonists, such as for example GW4064, 6α-ethylchenodeoxycholic acid (6-ECDCA), both of which are more potent activators than bile salts, AGN29, AGN31 or feraxamine [41, 42] have been developed. The list of synthetic agonists is rapidly growing (e.g. [45-47]), which is certainly fostered by the detailed structural knowledge of FXR [48]. As the canalicular exit step is rate limiting in hepatocellular handling of bile salts, BSEP is also subject to efficient short term regulation. Both, alterations of the carrier density of BSEP in the canalicular membrane via endo- and exocytosis as well as by phosphorylation state of BSEP control the secretory capacity of the canalicular membrane for bile salts [49, 50].

The main constituent of biliary lipids is phosphatidylcholine. Canalicular phospholipid secretion is critically dependent on MDR3 (ABCB4) [51]. This ABC transporter acts as a phosphatidylcholine translocator from the inner to the outer hemileaflet of the canalicular membrane [52]. From there, phosphatidylcholine is released into bile by the detergent action of bile salts secreted by BSEP [53]. In the canaliculus, phosphatidylcholine and bile salts form mixed micelles, which act as acceptors for poorly water-soluble substances, e.g. cholesterol [53]. Thereby, the release of cholesterol from the canalicular membrane into the aqueous canalicular lumen is facilitated by the heterodimeric ABC transporter ABCG5/ABCG8 [54]. Taken together, canalicular lipid secretion requires the coordinated action of the three ABC transporters BSEP, MDR3 and ABCG5/ABCG8. Furthermore and importantly, the mixed micelles formed in the canaliculi are instrumental to lower the toxicity of the high concentrations of bile salts in bile and by doing so prevent injury of the bile ducts by bile salts [55]. In addition, the canalicular membrane is rich in sphingomyelin [56], which associates with cholesterol [57, 58]. Such an

association was also observed in plasma membrane microdomains, so called lipid rafts [59, 60]. Lipid microdomains are resistant to solubilization by some detergents. Recently, lipid microdomains have been demonstrated in rat canalicular membrane [61]. Such microdomains might hence constitute a structural element to protect the canalicular membrane from the detergent action of high concentrations of bile salts in the canaliculi. As a further protective element, the canalicular membrane contains the P-type ATPase ATP8B1 (FIC1). This ATPase is thought to translocate phosphatidylserine from the outer to the inner leaflet of the canalicular membrane and thereby contributing to the resistance of the canalicular membrane towards solublization by bile salts [62-64].

Biliary organic anions other than bile salts mainly include bilirubin diglucuronide and glutathione. In addition, drug metabolites are secreted in anionic form as glucuronides or sulfates into bile. Two ABC transporters located in the canalicular plasma membrane are critically involved in canalicular metabolite secretion: multidrug resistance protein 2 (MRP2, ABCC2) has a preference for glucuronidated compounds, the prototypical substrate being bilirubin diglucuronide [65]. ABCG2 (also called breast cancer resistance protein or BCRP) mediates secretion of a variety of metabolites of endogenous and exogenous compounds and seems to have some bias towards sulfated compounds [66]. In addition, it is an important transporter conferring for tumor cells resistance to chemotherapy in cancer.

In summary, bile formation is an important function of the liver. For production of bile, hepatocytes are equipped with an array of basolateral uptake systems and ABC transporters in the canalicular membrane. These ABC transporters are able to export their substrates against large concentration gradients into the canaliculi, from where they enter the biliary tree. In the case of bile salt secretion, which is mediated by BSEP, a correct interplay between several ABC transporters is necessary to counteract the toxic activity of bile salts towards the bile ducts. Any interference with this process, either caused by inherited or acquired functional impairment of these transporters can potentially lead to pathophysiologic and hence to clinically relevant liver diseases.

INHERITED CHOLESTATIC LIVER DISEASE

In humans, many insights into the role of canalicular ABC transporters in bile formation and normal liver physiology were gained from inherited forms of cholestatic liver disease. Progressive familial intrahepatic cholestasis (PFIC) is a very rare form of liver disease, which clinically presents in three distinct entities. The prominent clinical manifestations of these diseases are cholestasis, pruritus and jaundice. Severe forms become clinically manifest early in childhood and usually require liver transplantation [67]. The molecular identification of the underlying genetic defects allows now a clear diagnostic distinction between the three forms: PFIC1 is caused by mutations in the gene coding for FIC1 (ATP8B1), PFIC2 by mutations in the gene for BSEP (ABCB11) and PFIC3 in the gene for MDR3 (ABCB4) [68]. As this review is focused on BSEP, only the pathophysiology of PFIC2 will be discussed. PFIC2 also presents with a milder form with recurring episodes of cholestasis, which is known as benign recurrent intrahepatic cholestasis type 2 or BRIC2. PFIC2 and BRIC2 can also be called BSEP deficiency syndrome [12]. Both PFIC2 and BRIC2 patients have mutations in the *BSEP* gene [69, 70]. Taken together, mutations in the *BSEP* gene are resulting in BSEP deficiency syndrome, which is a continuum from mild to severe, often progressive forms of intrahepatic cholestasis [12, 71, 72].

Frequently occurring mutations in the *BSEP* gene causing PFIC2 are missense mutations, nonsense mutations, deletions, insertions and splice site mutations. Patients with severe PFIC2 have less than 1 % of the normal amount of primary bile salts in their bile and are negative for BSEP staining with antibodies in their liver biopsies [73]. Recently, a comprehensive study reported a detailed analysis of patients with severe BSEP deficiency syndrome (PFIC2) from 109 families and identified 82 different mutations [74]. The mutations in these patients cluster in the two nucleotide binding domains of BSEP protruding into the cytoplasm. The BSEP nucleotide binding domains show a high degree of conservation between different species [49], which illustrates the importance of a correct structure of these domains for proper functioning of ABC-transporters [75-77]. The pathophysiologic consequences of the different mutations were studied by BSEP immunohistochemistry in biopsies, where available [74]. The vast majority of patients had abnormal or absent BSEP immunostaining, with a complete lack of BSEP staining being dominating. Hence, mutations in the *BSEP* gene tend to lead to absent BSEP expression in patients, which may be a consequence of the quality control system of protein synthesis in the endoplasmic reticulum (reviewed in [21]). Direct experimental evidence for this hypothesis is at the moment not available, since no comprehensive studies on mRNA levels in liver biopsies from patients with BSEP deficiency syndrome have been published to date. The number of novel mutations described for the *BSEP* gene is still increasing [78, 79]. An elegant follow up investigation of *BSEP* mutations studied the consequences of mutations and single-nucleotide polymorphisms in the *BSEP* gene from histologically characterized patients [74] on pre-mRNA splicing and on the subsequent processing of BSEP protein *in vitro* [80]. The authors identified 20 mutations/SNPs, which displayed reduced wild-type splicing and therefore reduced levels of normal mRNA in an *in vitro* system. Expression in CHO-K1 cells led for the majority of mutations to BSEP protein retention in the endoplasmic reticulum and subsequent degradation. This finding explains the lack of BSEP immunostaining in liver biopsies of such patients. In both studies, two common mutations were remarkable: The E297G and D482G mutants of BSEP varied most in their expression level among the respective carriers [74]. Furthermore, the common European mutant D482G displayed an enhanced aberrant splicing [80], which possibly provides an explanation for the wide variation of BSEP expression observed in patients with D482G mutations. Patients carrying this mutation have clinical phenotypes with variable severity, indicating a potential contribution of additional, yet unknown host factors to the pathogenesis of liver disease. Very recently, patients who developed antibodies against BSEP after liver transplantation for end-stage PFIC2 were reported [81, 82].

Such patients also develop persistent or transient severe BSEP deficiency syndrome. While one of these patients died, the others could be rescued by a more aggressive immunosuppressive regimen. This constitutes potentially a novel form of BSEP deficiency syndrome. However, it should be pointed out that the exact time point of the beginning of the (auto)immune reaction could not be determined in these patients.

Based on the clinical picture of severe BSEP deficiency syndrome, it can be concluded that BSEP is the only canalicular export system for bile salts. Unfortunately, information on functional consequences of mutant human BSEP forms can currently not be obtained from *in vivo* studies. Therefore, mutant and wild type forms of BSEP are functionally studied in heterologous expression systems. Using the Sf9 cell expression system, no substrate overlap between Bsep and the additional canalicular anion exporter Mrp2 was found [35]. Hence, also in *in vitro* studies, Mrp2 can not compensate for the loss of functional Bsep. Originally, seven BSEP mutations leading to severe BSEP deficiency syndrome, all affecting highly conserved amino acids, were studied in heterologous expression systems. These human mutations were introduced into the corresponding conserved positions of rat Bsep and subsequently expressed in MDCK cells [83]. Five of the studied mutations resulted in altered targeting of Bsep to the apical membrane, and mutant forms with reduced transport activity were identified. Hence, in principle information on the *in vivo* phenotype can be obtained from *in vitro* experiments [84]. However, in one study, the common D482G mutation displayed reduced transport activity [83], while the same mutation after being cloned into mouse Bsep displayed normal function [85]. Later, characterization of the D482G mutant BSEP form in HEK293 cells led to normal transport activity [86]. Also for the E297G variant, conflicting data on transport activity have been published [86, 87]. These discrepancies can potentially be explained by species differences of the Bsep backbone, differences in the interaction of BSEP with the membrane of the expression system or differences in RNA stability of this BSEP mutant in the different expression system. The latter should in principle not affect the results, as expression level of BSEP is controlled by Western blotting. Hence, while heterologous expression of BSEP is necessary to investigate the functional consequences of BSEP mutations, the results may be impacted by the choice of the expression systems and potentially also methodological differences between different labs and therefore have to be used with caution to understand clinical phenotypes of *BSEP* mutations.

Absent BSEP or BSEP lacking proper functional properties will lead to accumulation of bile salts within hepatocytes. Enhanced metabolism of bile salts within hepatocytes is a protective mechanism and leads to sulfated and glucuronidated bile salts [88, 89]. These metabolites are excreted into bile via the multidrug resistance protein MRP2 (ABCC2) [65], or back into the sinusoids by MRP3 (ABCC3) and MRP4 (ABCC4) [90], two salvage systems which help to reduce potentially cytotoxic intracellular bile salts. Recently, the heterodimeric organic solute transporter OSTα-OSTβ was also found to be expressed in the basolateral hepatocyte membrane [91]. OSTα-OSTβ is the major efflux transporter for bile salts from the enterocytes in the ileum into the blood and therefore crucial for the reclaiming of bile salts from the small intestine [8]. It is therefore conceivable that OSTα-OSTβ could also act as a basolateral salvage (efflux) system for bile salts. However, the relative contribution of these three adaptive basolateral bile salt salvage systems is currently not fully understood and needs to be worked out in detail. Despite these adaptive responses, hydrophobic bile salts will with time damage hepatocytes resulting in portal inflammation and giant-cell hepatitis. In addition, observations from patients with severe forms of BSEP deficiency syndrome have revealed that they are already at very young age at significant risk to develop hepatocellular carcinoma [74, 92]. The detailed mechanisms of bile salt induced cell transformation is not known in detail, but bile salt induced carcinogenesis may involve mitochondria [93, 94], interference with signaling cascades controlling cell cycle [95] and/or activation of homeobox genes [96]. Furthermore, it seems conceivable that elevated intracellular bile salt levels could interfere with DNA repair mechanisms.

ACQUIRED CHOLESTATIC LIVER DISEASE

A rather evident form of acquired cholestatic liver disease is bile duct obstruction caused by gall stones or tumors. It leads to extrahepatic cholestasis. In contrast, inhibition of BSEP by xenobiotics such as drugs or endogenous metabolites will lead to reduced canalicular bile salt secretion and consequently to intracellular accumulation of bile salts. If persistent, this will lead to clinical signs of intrahepatic cholestasis and ultimately to liver injury. Drug induced liver injury including cholestasis is a frequent clinical entity, which leads to a considerable number of hospital admissions and in severe cases requires liver transplantation [97]. Furthermore, adverse drug reactions resulting in liver injury are an important cause for attrition of drugs during development or for withdrawal of drugs from the market [98, 99]. It was found in medical inpatients that about 30 % of drug induced liver injuries are cholestatic or mixed [100].

Examples of drugs known to cause cholestasis in susceptible individuals are cyclosporine, rifampicin, rifamycin, glibenclamide or bosentan. Using the insect cell expression system, these drugs have been found to be competitive inhibitors of rat and human BSEP [35, 101, 102]. The endothelin receptor antagonist bosentan is an example of a drug, where the cholestatic potential has been worked out in detail. Bosentan is taken up by OATP1B1 and OATP1B3 into hepatocytes and its metabolites sequestered for biliary excretion, which is the main elimination route for bosentan [103]. Bosentan caused asymptomatic, reversible transaminase elevations in some patients, while being evaluated in clinical trials [104]. In these patients, plasma bile salt levels increased with increasing dose of bosentan. As a conesquence of competitive inhibition of [102, 104], bosentan treatment of rats led to an elevation of plasma bile salt levels. This elevation of bile salts was more pronounced under coadministration of glibenclamide [104]. These findings strongly suggest that in patients *in vivo* bosentan acts as a competitive, specific BSEP inhibitor, since no elevation of serum bilirubin was found [104]. The list of Bsep/BSEP inhibitors is continuously growing [105]. Further investiga-

tion of the mechanism of bosentan-induced cholestasis in rats revealed that bosentan leads to a stimulation of bile flow [106]. This is somewhat unexpected since the pathophysiologic hallmark of cholestasis is a reduction of bile flow. However, bosentan did not affect biliary bile salt output. In combination with the increased bile salt-independent bile flow, this will lead to a lower bile salt concentration in canalicular bile concomitant with a reduced biliary lipid secretion. This in turn might negatively affect canalicular lipid composition and lipid asymmetry and hence contribute to intracellular accumulation of bile salts and other cholephilic substances [107]. The choleretic effect of bosentan was dependent on the presence of functional Mrp2 [106]. In an additional *in vitro* study with the Sf9 cell system, bosentan mediated inhibiton of rat and human BSEP was confirmed [108]. Importantly, the same study demonstrated a stimulation of rat and human MRP2 transport activity by bosentan and therefore offers a mechanistic explanation of the observed increase of bile salt-independent bile flow in rats.

Another form of a mixed genetic and acquired cholestasis is intrahepatic cholestasis of pregnancy [109]. Cholestasis of pregnancy often starts in the third trimester and is characterized by pruritus, and elevated serum bile salts and transaminases [109-112]. After delivery, cholestasis rapidly resolves. Findings of elevated serum levels of steroids during pregnancy link estrogen and its metabolites [113, 114] and progesterone and its metabolites to the pathogenesis of cholestasis of pregnancy [115-117]. This link is also supported by the observation of oral contraceptive induced cholestasis [114, 118]. Mice with a disrupted gene for the estrogen receptor α are insensitve to induction of cholestasis by estradiol treatment, indicating a key role of this receptor for the pathogenesis of estrogen induced cholestasis [119]. In rats, application of pharmacological doses of estradiol-17β-glucuronide or progesterone sulfate has been found to cause acute cholestasis [120, 121]. Of note, estradiol-17β-glucuronide induced cholestasis in rats is strictly dependent on the expression of Mrp2 in the canalicular membrane [122]. Therefore, estradiol-17β-glucuronide was tested on rat Bsep expressed in Sf9 cell vesicles, but did not inhibit ATP-dependent taurocholate transport [35]. However, using Sf9 cell membrane vesicles expressing both, Bsep and Mrp2, estradiol-17β-glucuronide inhibited taurocholate transport in a time- and dose-dependent manner [35]. It was concluded from this finding that Bsep is indirectly inhibited and this inhibition was postulated to reflect a trans-inhibition, i.e. estradiol-17β-glucuronide needs to be secreted into the canalicular lumen for Bsep inhibition [35]. An alternate explanation could be a physical interaction of Bsep and Mrp2 in the membrane, mediated by estradiol-17β-glucuronide [122]. Indirect inhibition of Bsep by estradiol-17β-glucuronide was confirmed and extended to sulfated progesterone metabolites [121, 123] and also reported for the HER1/HER2 inhibitor PKI166 [124].

In summary, drug inhibition of BSEP followed by acquired cholestasis is now well established. The exact mechanism of BSEP inhibition is drug specific and can either be direct (competitive) or indirect. In the latter case, inhibition likely occurs from the side of the canaliculus and requires in addition MRP2. Finally, drug activation of MRP2 leading to an increase of bile salt-independent bile flow may aggravate the cholestatic potential of a drug. In addition, acute experiments with estradiol-17β-glucuronide in rats have demonstrated that in less than 30 minutes a rapid internalization of part of Mrp2 and Bsep into a subapical, vesicular compartment occurs [125-127]. This internalization is dependent on Ca^{2+}-dependent protein kinase C [128] and will consequently lead to a reduction of canalicular bile flow.

BSEP AS A POTENTIAL THERAPEUTIC TARGET

The general principle of therapeutic intervention in cholestatic liver disease is to restore or to enhance canalicular bile formation and consequently to lower intracellular load of hydrophobic bile salts in hepatocytes. Normal canalicular bile flow might also contribute to normalization of canalicular lipid composition. An additional benefit of increased canalicular bile formation is a lowering of the toxic potential of biliary bile salts to the bile ducts. In addition, induction of metabolism of hydrophobic bile salts as well as induction of the basolateral salvage systems is also a therapeutic option. An additional aspect of the treatment of cholestatic disorders is improvement of the quality of life. Prolonged cholestasis is often accompanied by severe pruritus, a condition having a severe negative impact on affected patients. Therefore, measures reducing this symptom, even if they are not able to cure patents, are highly needed and frequently used.

As there are multiple mechanism of BSEP related cholestasis, a variety of options are available to the physician. In obstructive forms of cholestasis, endoscopic or surgical removal of the obstruction or restoration of bile flow by endoscopic (or percutaneous) insertion of a stent into the obstructed bile duct is the standard procedure. It has however to be kept in mind that in particular insertion of a stent is not a cure, as it does not tackle the cause of the obstruction. In cases of drug induced cholestasis, discontinuation of the drug or lowering the dose of the drug is indicated. A good example for this is bosentan, where serum bile salt levels of the affected patients turned quickly back to normal after discontinuation of the drug [129]. However, in cases where additional mechanisms of liver injury are important, removal of the drug may not be sufficient. An example for this is the antidiabetic drug troglitazone, which was withdrawn from the market due to liver toxicity. While the exact mechanism of toxicity is not fully understood, mitochondrial toxicity of troglitazone is clearly relevant [130, 131]. In addition, troglitazone and its main metabolite are clearly cholestatic [132, 133]. In many patients, onset of liver injury was delayed and/or persisted after discontinuation of troglitazone [131]. In addition, patients with diabetes often suffer from non-alcoholic steatohepatitis, which may further complicate the disease course.

The situation with inherited defects of BSEP malfunction is very different. Gene therapy for such patients is currently not an option and it is very hard to predict at this moment, when it will become available. In case of non-functional BSEP mutants, therapeutic options are very limited. The main goals are to restore as much as possible canalicular bile salt secretion and to lower intracellular bile salt load, and hence prevent or lower the risk for fibrosis/cirrhosis and and neoplastic transformations [134]. The latter point is important as PFIC2 patients have a considerable risk for

developing hepatocellular carcinoma at young age [74, 92]. While ursodeoxycholate (UDC) has proved to be a valuable option for patients with PFIC3, patients with PFIC2 show a mixed response to treatment with this bile salt [135]. UDC shifts the bile salt pool from more toxic hydrophobic bile salts to less toxic hydrophilic bile salts [136] and has been shown to upregulate canalicular transporter synthesis and membrane insertion at the post-transcriptional level [50, 137-140] and consequently stimulates bile flow. Thus, an adequate BSEP function once BSEP is inserted into its target apical membrane is of key importance for UDC to exert its anticholestatic effect. As BSEP structure and function are often compromised in PFIC2, it is not surprising that UDC is of limited therapeutic value for PFIC2. The C23 homologue of C24-UDC, norUDC, has been demonstrated in an experimental mouse model of cholestasis to exert strong anticholestatic and antifibrotic effects [141]. Studies on its efficacy in human cholangiopathies are awaited in the near future.

Pruritus is a major burden for patients with PFIC2. Its pathogenesis is yet unresolved [142]. The non-absorbable anion exchanger resin cholestyramine has been proposed for the symptomatic treatment of pruritus in cholestatic disorders of different origin and is, due to its low rate of serious side effects, widely used for this purpose at least temporarily [143]. However, evidence for its antipruritc efficacy from well performed, controlled studies is lacking [144]. Rifampicin is a potent antipruritic agent in cholestasis [143, 144]. In addition, rifampicin may abort cholestatic episodes in BRIC2 [134]. It is known to induce hydroxylation of hydrophobic bile salts in hepatocytes allowing them to be glucuronidated and consequently to lower the cytotoxic potential of these bile salts in cholestatic conditions [139]. Thus, rifampicin represents a therapeutic option for the medical treatment of pruritus in PFIC2 as well as for BRIC2.

FXR is a bile salt activated transcription factor that plays a critical role in bile salt homeostasis [145, 146]. FXR reduces the body load of bile salts by decreasing their biosynthesis (CYP7A1 (CYP, cytochrome P450) ↓, CYP8B1 ↓), and increasing their metabolism (CYP3A4 ↑, UGT2B4 ↑ (UGT, uridine diphosphate glucuronosyltransferase) UGT2B7 ↑) and hepatic (BSEP ↑) and intestinal (apical sodium dependent bile acid transporter, ASBT or SLC10A2 ↓) elimination. Based on these observations, FXR ligands may offer a rational treatment option for cholestatic liver diseases. The selective FXR agonist 6-ECDCA prevented bile flow impairment induced by lithocholic acid (LCA) or 17α-ethinylestradiol (E_2-17α) in rat models of cholestasis and protected hepatocytes against acute necrosis caused by LCA [147, 148]. *In vivo* administration of 6-ECDCA in rats led to enhanced expression of Bsep, Mrp2, Mdr2, and Shp, while it repressed Cyp7a1, Cyp8b1 and Ntcp mRNA expression. The first phase II pilot trial of 6-ECDCA in patients with primary biliary cirrhosis has recently been performed [149] and results are eagerly awaited. Stimulation of adaptive response to cholestasis by FXR agonists is likely to be beneficial in canalicular cholestasis, where secretory failure of hepatocytes is the cause in early partial/incomplete obstruction. In addition, an antifibrotic effect of FXR agonists has also been described [150].

Treatment options of pruritus in cholestatic disorders [142] include therapy of the underlying disease with UDC (13-15 mg/kg/d) when appropriate, the anion exchange resin cholestyramine (1-2 x 4g/d), the pregnane X receptor (PXR, NR1I2) agonist and enzyme inducer rifampicin (2 x 150 mg/d, max. 2 x 300 mg/d; cave: hepatitis after 6-12 weeks in ≤12%), the opioid antagonist, naltrexone (25-50 mg/d; cave: initial opioid withdrawal symptoms), and the serotonine reuptake inhibitor, sertraline (75 mg/d) [143].

UDC has been shown to be an effective therapy of pruritus in intrahepatic cholestasis of pregnancy, but not in primary biliary cirrhosis (PBC) [143]. However, no studies have so far been designed to selectively test the effect of UDC on pruritus in PBC or primary sclerosing cholangitis (PSC) as a primary endpoint. Circumstantial evidence rather than adequately performed randomized, placebo-controlled trials showed that cholestyramine exerts antipruritic effects in PBC. The PXR agonist and enzyme inducer rifampicine has been shown to effectively diminish pruritus in PBC and to be superior to the constitutive androgen receptor (CAR, NR1I3) agonist and enzyme inducer phenobarbital. The opioid receptor antagonist naltrexone may diminish pruritus when administered at doses of 25-50 mg/d (note: start with very low doses to prevent opioid withdrawal-like symptoms) and is easier to administer than the subcutaneously applied naloxone. The serotonin reuptake inhibitor, sertraline, induced some relief of pruritus in a randomized, placebo-controlled crossover study. Experimental and therapeutic approaches in otherwise treatment-resistant, desperate patients include plasmapheresis, albumin dialysis, plasma separation and anion absorption, nasobiliary drainage, biliary diversion and liver transplantation [143].

So far, treatment options for severe forms of PFIC2 are limited to partial biliary diversion and liver transplantation [143]. On two patients, hepatocyte transplantation was performed, but both needed a subsequent liver transplantation [151, 152]. However, as outlined above, some BSEP mutants display residual transport activity. A patient with benign recurrent intrahepatic cholestasis type 2 was identified as a carrier of combined heterozygous BSEP mutations (p.E297G and p.R432T) [87]. *In vitro* characterization of these mutations showed a massive reduction of their v_{max} compared to wild type BSEP. Hence, it may be possible to have a normal or less impaired liver function with less than normal BSEP in the canalicular membrane. In addition, in a cohort of 110 healthy liver samples, BSEP expression levels varied widely [153], again suggesting that less than normal BSEP levels lead to a sufficient bile secretory capacity of the liver. A further investigation of BSEP mutants in the MDCK cell expression system demonstrated that the E297G and the D482G mutant of BSEP reach the apical target membrane with a massively reduced efficiency compared to wild type BSEP [86]. Treating MCDK cells expressing these two mutants with 4-phenylbutyrate led to an increase of the BSEP mutants in the apical membrane of MDCK cells [154]. As both variants display at least residual transport activity, this finding may open a possibility for a pharmacologic treatment of some forms of inherited BSEP deficiency syndrome. Such an approach has become feasible now as it was recently demonstrated in three patients with ornithine transcarbamylase deficiency that treatment with 4-phenylacetate, a

metabolite of 4-phenylbutyrate [155] BSEP expression is upregulated at the protein, but not at the mRNA level [156].

CONCLUSION

Progress has been enormous in understanding physiology and pathophysiology of enterohepatic circulation of bile salts from the earliest concepts to the current understanding of molecular mechanisms of impaired BSEP function. While clear concepts on the pathogenesis of inherited and acquired forms of BSEP deficiency syndrome exist, therapeutic options are still limited and consist mainly of interventional procedures such as biliary diversion or liver transplantation [135]. Drug treatment has so far been mainly symptomatic with the exception of bland acquired cholestasis, where discontinuation of the insulting agent will usually cure the patient. Starting from the established interventions using ursodoexycholate and/or inducers of liver detoxification, identification of novel drugs for treating cholestatic liver disease may lure around the corner.

ACKNOWLEDGEMENT

Bruno Stieger is supported by grant # 31003A 124652 / 1 from the Swiss National Science Foundation.

REFERENCES

[1] Esteller A. Physiology of bile secretion. World J Gastroenterol 2008; 14: 5641-9.
[2] Trauner M, Boyer JL. Bile salt transporters: molecular characterization, function, and regulation. Physiol Rev 2003; 83: 633-71.
[3] Russell DW. Fifty years of advances in bile acid synthesis and metabolism. J Lipid Res 2009; 50 Suppl: S120-5.
[4] Hunt MC, Alexson SE. Novel functions of acyl-CoA thioesterases and acyltransferases as auxiliary enzymes in peroxisomal lipid metabolism. Prog Lipid Res 2008; 47: 405-21.
[5] Hofmann AF, Hagey LR. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. Cell Mol Life Sci 2008; 65: 2461-83.
[6] Hofmann AF. Bile acids: trying to understand their chemistry and biology with the hope of helping patients. Hepatology 2009; 49: 1403-18.
[7] Hofmann AF. The enterohepatic circulation of bile acids in mammals: form and functions. Front Biosci 2009; 14: 2584-98.
[8] Dawson PA, Lan T, Rao A. Bile acid transporters. J Lipid Res 2009; 50: 2340-57.
[9] Beuers U, Boyer JL. Bile - a historical review of studies on its form and function. In: Kirsner JB, editor. Gastroenterology in the 20th century. New York: Lea & Ferbiger; 1994. p. 267-88.
[10] Meier PJ, Stieger B. Bile salt transporters. Annu Rev Physiol 2002; 64:635-61.
[11] Kullak-Ublick GA, Stieger B, Meier PJ. Enterohepatic bile salt transporters in normal physiology and liver disease. Gastroenterology 2004; 126: 322-42.
[12] Pauli-Magnus C, Stieger B, Meier Y, Kullak Ublick GA, Meier PJ. Enterohepatic transport of bile salts and genetics of cholestasis. J Hepatol 2005; 43: 342-57.
[13] Krahenbuhl S, Talos C, Fischer S, Reichen J. Toxicity of bile acids on the electron transport chain of isolated rat liver mitochondria. Hepatology 1994; 19: 471-9.
[14] Rust C, Wild N, Bernt C, Vennegeerts T, Wimmer R, Beuers U. Bile acid-induced apoptosis in hepatocytes is caspase-6-dependent. J Biol Chem 2009; 284: 2908-16.
[15] Hagenbuch B, Dawson P. The sodium bile salt cotransport family SLC10. Pflugers Arch 2004; 447: 566-70.
[16] Hagenbuch B, Meier PJ. Organic anion transporting polypeptides of the OATP/*SLC21* family: Phylogenetic classification as OATP/*SLCO* superfamily, new nomenclature and molecular/ functional properties. Pflugers Arch 2004; 447: 653-65.
[17] Hagenbuch B, Gui C. Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family. Xenobiotica 2008; 38: 778-801.
[18] Stolz A, Takikawa H, Ookhtens M, Kaplowitz N. The role of cytoplasmic proteins in hepatic bile acid transport. Annu Rev Physiol 1989; 51: 161-76.
[19] Agellon LB, Torchia EC. Intracellular transport of bile acids. Biochim Biophys Acta 2000; 1486: 198-209.
[20] Stieger B, Meier Y, Meier PJ. The bile salt export pump. Pflugers Arch 2007; 453: 611-20.
[21] Stieger B. Recent insights into the function and regulation of the bile salt export pump (ABCB11). Curr Opin Lipidol 2009; 20: 176-81.
[22] Reichen J, Paumgartner G. Uptake of bile acids by perfused rat liver. Am J Physiol 1976; 231: 734-42.
[23] Hofmann AF, Mysels KJ. Bile acid solubility and precipitation *in vitro* and *in vivo*: the role of conjugation, pH, and Ca2+ ions. J Lipid Res 1992; 33: 617-26.
[24] Weinman SA, Maglova LM. Free concentrations of intracellular fluorescent anions determined by cytoplasmic dialysis of isolated hepatocytes. Am J Physiol 1994; 267: G922-31.
[25] Setchell KD, Rodrigues CM, Clerici C, *et al*. Bile acid concentrations in human and rat liver tissue and in hepatocyte nuclei. Gastroenterology 1997; 112: 226-35.
[26] Masyuk AI, LaRusso NF. Aquaporins in the hepatobiliary system. Hepatology 2006; 43 (2 Suppl 1): S75-81.
[27] Geyer J, Wilke T, Petzinger E. The solute carrier family SLC10: more than a family of bile acid transporters regarding function and phylogenetic relationships. Naunyn Schmiedebergs Arch Pharmacol 2006; 372: 413-31.
[28] Alrefai WA, Gill RK. Bile acid transporters: structure, function, regulation and pathophysiological implications. Pharm Res 2007; 24: 1803-23.
[29] Ho RH, Tirona RG, Leake BF, *et al*. Drug and bile acid transporters in rosuvastatin hepatic uptake: function, expression, and pharmacogenetics. Gastroenterology 2006; 130: 1793-806.
[30] Eloranta JJ, Kullak-Ublick GA. The role of FXR in disorders of bile acid homeostasis. Physiology (Bethesda) 2008; 23: 286-95.
[31] Anwer MS. Cellular regulation of hepatic bile acid transport in health and cholestasis. Hepatology 2004; 39: 581-90.
[32] Kalliokoski A, Niemi M. Impact of OATP transporters on pharmacokinetics. Br J Pharmacol 2009; 158: 693-705.
[33] Glavy JS, Wu SM, Wang PJ, Orr GA, Wolkoff AW. Down-regulation by extracellular ATP of rat hepatocyte organic anion transport is mediated by serine phosphorylation of oatp1. J Biol Chem 2000; 275: 1479-84.
[34] Guo GL, Klaassen CD. Protein kinase C suppresses rat organic anion transporting polypeptide 1- and 2-mediated uptake. J Pharmacol Exp Ther 2001; 299: 551-7.
[35] Stieger B, Fattinger K, Madon J, Kullak Ublick GA, Meier PJ. Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. Gastroenterology 2000; 118: 422-30.
[36] Hayashi H, Takada T, Suzuki H, Onuki R, Hofmann AF, Sugiyama Y. Transport by vesicles of glycine- and taurine-conjugated bile salts and taurolithocholate 3-sulfate: a comparison of human BSEP with rat Bsep. Biochim Biophys Acta 2005; 1738: 54-62.
[37] Gerloff T, Stieger B, Hagenbuch B, *et al*. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. J Biol Chem 1998; 273: 10046-50.
[38] Carlton VE, Harris BZ, Puffenberger EG, *et al*. Complex inheritance of familial hypercholanemia with associated mutations in TJP2 and BAAT. Nat Genet 2003; 34: 91-6.
[39] Hirano M, Maeda K, Hayashi H, Kusuhara H, Sugiyama Y. Bile salt export pump (BSEP/ABCB11) can transport a nonbile acid substrate, pravastatin. J Pharmacol Exp Ther 2005; 314: 876-82.
[40] Lo Sasso G, Petruzzelli M, Moschetta A. A translational view on the biliary lipid secretory network. Biochim Biophys Acta 2008; 1781: 79-96.
[41] Fiorucci S, Rizzo G, Donini A, Distrutti E, Santucci L. Targeting farnesoid X receptor for liver and metabolic disorders. Trends Mol Med 2007; 13: 298-309.
[42] Wang YD, Chen WD, Moore DD, Huang W. FXR: a metabolic regulator and cell protector. Cell Res 2008; 18: 1087-95.

[43] Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile Acid receptors in metabolic regulation. Physiol Rev 2009; 89: 147-91.

[44] Del Bas JM, Ricketts ML, Vaque M, *et al*. Dietary procyanidins enhance transcriptional activity of bile acid-activated FXR *in vitro* and reduce triglyceridemia *in vivo* in a FXR-dependent manner. Mol Nutr Food Res 2009; 53: 805-14.

[45] Mehlmann JF, Crawley ML, Lundquist JTt, *et al*. Pyrrole[2,3-d]azepino compounds as agonists of the farnesoid X receptor (FXR). Bioorg Med Chem Lett 2009; 19: 5289-92.

[46] Fiorucci S, Mencarelli A, Distrutti E, Palladino G, Cipriani S. Targetting farnesoid-X-receptor: from medicinal chemistry to disease treatment. Curr Med Chem 2010; 17: 139-59.

[47] Lundquist JT, Harnish DC, Kim CY, *et al*. Improvement of Physiochemical Properties of the Tetrahydroazepinoindole Series of Farnesoid X Receptor (FXR) Agonists: Beneficial Modulation of Lipids in Primates. J Med Chem 2010; 53: 1774-87.

[48] Mi LZ, Devarakonda S, Harp JM, *et al*. Structural basis for bile acid binding and activation of the nuclear receptor FXR. Mol Cell 2003; 11: 1093-100.

[49] Noe J, Hagenbuch B, Meier PJ, St-Pierre MV. Characterization of the mouse bile salt export pump overexpressed in the baculovirus system. Hepatology 2001; 33: 1223-31.

[50] Beuers U. Drug Insight: mechanisms and sites of action of ursodeoxycholic acid in cholestasis. Nat Clin Pract Gastroenterol Hepatol 2006; 3: 318-28.

[51] Smit JJ, Schinkel AH, Oude Elferink RP, *et al*. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell 1993; 75: 451-62.

[52] Oude Elferink RP, Paulusma CC. Function and pathophysiological importance of ABCB4 (MDR3 P-glycoprotein). Pflugers Arch 2007; 453: 601-10.

[53] Small DM. Role of ABC transporters in secretion of cholesterol from liver into bile. Proc Natl Acad Sci USA 2003; 100: 4-6.

[54] Hazard SE, Patel SB. Sterolins ABCG5 and ABCG8: regulators of whole body dietary sterols. Pflugers Arch 2007; 453: 745-52.

[55] Trauner M, Fickert P, Halilbasic E, Moustafa T. Lessons from the toxic bile concept for the pathogenesis and treatment of cholestatic liver diseases. Wien Med Wochenschr 2008; 158: 542-8.

[56] Gerloff T, Meier PJ, Stieger B. Taurocholate induces preferential release of phosphatidylcholine from rat liver canalicular vesicles. Liver 1998; 18: 306-12.

[57] van Erpecum KJ, Carey MC. Influence of bile salts on molecular interactions between sphingomyelin and cholesterol: relevance to bile formation and stability. Biochim Biophys Acta 1997; 1345: 269-82.

[58] Nibbering CP, Carey MC. Sphingomyelins of rat liver: biliary enrichment with molecular species containing 16:0 fatty acids as compared to canalicular-enriched plasma membranes. J Membr Biol 1999; 167: 165-71.

[59] Munro S. Lipid rafts: elusive or illusive? Cell 2003; 115: 377-88.

[60] Rajendran L, Simons K. Lipid rafts and membrane dynamics. J Cell Sci 2005; 118: 1099-102.

[61] Ismair MG, Hausler S, Stuermer CA, *et al*. ABC-transporters are localized in caveolin-1-positive and reggie-1-negative and reggie-2-negative microdomains of the canalicular membrane in rat hepatocytes. Hepatology 2009; 49: 1673-82.

[62] Paulusma CC, Groen A, Kunne C, *et al*. Atp8b1 deficiency in mice reduces resistance of the canalicular membrane to hydrophobic bile salts and impairs bile salt transport. Hepatology 2006; 44: 195-204.

[63] Folmer DE, Elferink RP, Paulusma CC. P4 ATPases - lipid flippases and their role in disease. Biochim Biophys Acta 2009; 1791: 628-35.

[64] Cai SY, Gautam S, Nguyen T, Soroka CJ, Rahner C, Boyer JL. ATP8B1 deficiency disrupts the bile canalicular membrane bilayer structure in hepatocytes, but FXR expression and activity are maintained. Gastroenterology 2009; 136: 1060-9.

[65] Nies AT, Keppler D. The apical conjugate efflux pump ABCC2 (MRP2). Pflugers Arch 2007; 453: 643-59.

[66] Kusuhara H, Sugiyama Y. ATP-binding cassette, subfamily G (ABCG family). Pflugers Arch 2007; 453: 735-44.

[67] Davit-Spraul A, Gonzales E, Baussan C, Jacquemin E. Progressive familial intrahepatic cholestasis. Orphanet J Rare Dis 2009; 4: 1.

[68] Oude Elferink RP, Paulusma CC, Groen AK. Hepatocanalicular transport defects: pathophysiologic mechanisms of rare diseases. Gastroenterology 2006; 130: 908-25.

[69] Strautnieks SS, Bull LN, Knisely AS, *et al*. A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. Nat Genet 1998; 20: 233-8.

[70] van Mil SW, van der Woerd WL, van der Brugge G, *et al*. Benign recurrent intrahepatic cholestasis type 2 is caused by mutations in ABCB11. Gastroenterology 2004; 127: 379-84.

[71] Lam CW, Cheung KM, Tsui MS, Yan MS, Lee CY, Tong SF. A patient with novel ABCB11 gene mutations with phenotypic transition between BRIC2 and PFIC2. J Hepatol 2006; 44: 240-2.

[72] Takahashi A, Hasegawa M, Sumazaki R, *et al*. Gradual improvement of liver function after administration of ursodeoxycholic acid in an infant with a novel ABCB11 gene mutation with phenotypic continuum between BRIC2 and PFIC2. Eur J Gastroenterol Hepatol 2007; 19: 942-6.

[73] Jansen PLM, Strautnieks SS, Jacquemin E, *et al*. Hepatocanalicular bile salt export pump deficiency in patients with progressive familial intrahepatic cholestasis. Gastroenterology 1999; 117: 1370-9.

[74] Strautnieks SS, Byrne JA, Pawlikowska L, *et al*. Severe bile salt export pump deficiency: 82 different ABCB11 mutations in 109 families. Gastroenterology 2008; 134: 1203-14.

[75] Locher KP. Review. Structure and mechanism of ATP-binding cassette transporters. Philos Trans R Soc Lond B Biol Sci 2009; 364: 239-45.

[76] Seeger MA, van Veen HW. Molecular basis of multidrug transport by ABC transporters. Biochim Biophys Acta 2009; 1794: 725-37.

[77] Rees DC, Johnson E, Lewinson O. ABC transporters: the power to change. Nat Rev Mol Cell Biol 2009; 10: 218-27.

[78] Chen HL, Liu YJ, Su YN, *et al*. Diagnosis of BSEP/ABCB11 mutations in Asian patients with cholestasis using denaturing high performance liquid chromatography. J Pediatr 2008; 153: 825-32.

[79] Treepongkaruna S, Gaensan A, Pienvichit P, *et al*. Novel ABCB11 mutations in a Thai infant with progressive familial intrahepatic cholestasis. World J Gastroenterol 2009; 15: 4339-42.

[80] Byrne JA, Strautnieks SS, Ihrke G, *et al*. Missense mutations and single nucleotide polymorphisms in ABCB11 impair bile salt export pump processing and function or disrupt pre-messenger RNA splicing. Hepatology 2009; 49: 553-67.

[81] Keitel V, Burdelski M, Vojnisek Z, Schmitt L, Haussinger D, Kubitz R. De novo bile salt transporter antibodies as a possible cause of recurrent graft failure after liver transplantation: a novel mechanism of cholestasis. Hepatology 2009; 50: 510-7.

[82] Jara P, Hierro L, Martinez-Fernandez P, *et al*. Recurrence of bile salt export pump deficiency after liver transplantation. N Engl J Med 2009; 361: 1359-67.

[83] Wang L, Soroka CJ, Boyer JL. The role of bile salt export pump mutations in progressive familial intrahepatic cholestasis type II. J Clin Invest 2002; 110: 965-72.

[84] Lam P, Pearson CL, Soroka CJ, Xu S, Mennone A, Boyer JL. Levels of plasma membrane expression in progressive and benign mutations of the bile salt export pump (Bsep/Abcb11) correlate with severity of cholestatic diseases. Am J Physiol 2007; 293: C1709-16.

[85] Plass JR, Mol O, Heegsma J, *et al*. A progressive familial intrahepatic cholestasis type 2 mutation causes an unstable, temperature-sensitive bile salt export pump. J Hepatol 2004; 40: 24-30.

[86] Hayashi H, Takada T, Suzuki H, Akita H, Sugiyama Y. Two common PFIC2 mutations are associated with the impaired membrane trafficking of BSEP/ABCB11. Hepatology 2005; 41: 916-24.

[87] Noe J, Kullak-Ublick GA, Jochum W, *et al*. Impaired expression and function of the bile salt export pump due to three novel ABCB11 mutations in intrahepatic cholestasis. J Hepatol 2005; 43: 536-43.

[88] Alnouti Y. Bile Acid sulfation: a pathway of bile acid elimination and detoxification. Toxicol Sci 2009; 108: 225-46.

[89] Zollner G, Trauner M. Molecular mechanisms of cholestasis. Wien Med Wochenschr 2006; 156: 380-5.

[90] Geier A, Wagner M, Dietrich CG, Trauner M. Principles of hepatic organic anion transporter regulation during cholestasis, inflammation and liver regeneration. Biochim Biophys Acta 2007; 1773: 283-308.

[91] Ballatori N, Li N, Fang F, Boyer JL, Christian WV, Hammond CL. OST alpha-OST beta: a key membrane transporter of bile acids and conjugated steroids. Front Biosci 2009; 14: 2829-44.
[92] Knisely AS, Strautnieks SS, Meier Y, *et al*. Hepatocellular carcinoma in ten children under five years of age with bile salt export pump deficiency. Hepatology 2006; 44: 478-86.
[93] Palmeira CM, Rolo AP. Mitochondrially-mediated toxicity of bile acids. Toxicology 2004; 203: 1-15.
[94] Sokol RJ, Devereaux M, Dahl R, Gumpricht E. "Let there be bile"--understanding hepatic injury in cholestasis. J Pediatr Gastroenterol Nutr 2006; 43 Suppl 1: S4-9.
[95] Atherford PA, Jankowski JA. Molecular biology of Barrett's cancer. Best Pract Res Clin Gastroenterol 2006; 20: 813-27.
[96] Souza RF, Krishnan K, Spechler SJ. Acid, bile, and CDX: the ABCs of making Barrett's metaplasia. Am J Physiol 2008; 295:G211-8.
[97] Bleibel W, Kim S, D'Silva K, Lemmer ER. Drug-induced liver injury: review article. Dig Dis Sci 2007; 52: 2463-71.
[98] Schuster D, Laggner C, Langer T. Why drugs fail--a study on side effects in new chemical entities. Curr Pharm Des 2005; 11: 3545-59.
[99] Smith DA, Schmid EF. Drug withdrawals and the lessons within. Curr Opin Drug Discov Dev 2006; 9: 38-46.
[100] Meier Y, Cavallaro M, Roos M, *et al*. Incidence of drug-induced liver injury in medical inpatients. Eur J Clin Pharmacol 2005; 61: 135-43.
[101] Byrne JA, Strautnieks SS, Mieli-Vergani G, Higgins CF, Linton KJ, Thompson RJ. The human bile salt export pump: characterization of substrate specificity and identification of inhibitors. Gastroenterology 2002; 123: 1649-58.
[102] Noe J, Stieger B, Meier PJ. Functional expression of the canalicular bile salt export pump of human liver. Gastroenterology 2002; 123: 1659-66.
[103] Treiber A, Schneiter R, Hausler S, Stieger B. Bosentan is a substrate of human OATP1B1 and OATP1B3: inhibition of hepatic uptake as the common mechanism of its interactions with cyclosporin A, rifampicin, and sildenafil. Drug Metab Dispos 2007; 35: 1400-7.
[104] Fattinger K, Cattori V, Hagenbuch B, Meier PJ, Stieger B. Rifamycin SV and rifampicin exhibit differential inhibition of the hepatic rat organic anion transporting polypeptides, Oatp1 and Oatp2. Hepatology 2000; 32: 82-6.
[105] Stieger B. Role of the bile salt export pump, BSEP, in acquired forms of cholestasis. Drug Metab Disp 2010; 42: 437-45.
[106] Fouassier L, Kinnman N, Lefevre G, *et al*. Contribution of mrp2 in alterations of canalicular bile formation by the endothelin antagonist bosentan. J Hepatol 2002; 37: 184-91.
[107] Meier PJ. Canalicular bile formation: beyond single transporter functions. J Hepatol 2002; 37: 272-3.
[108] Mano Y, Usui T, Kamimura H. Effects of bosentan, an endothelin receptor antagonist, on bile salt export pump and multidrug resistance-associated protein 2. Biopharm Drug Dispos 2007; 28: 13-8.
[109] Geenes V, Williamson C. Intrahepatic cholestasis of pregnancy. World J Gastroenterol 2009; 15: 2049-66.
[110] Pusl T, Beuers U. Intrahepatic cholestasis of pregnancy. Orphanet J Rare Dis 2007; 2: 26.
[111] Hay JE. Liver disease in pregnancy. Hepatology 2008; 47: 1067-76.
[112] Gonzales E, Davit-Spraul A, Baussan C, Buffet C, Maurice M, Jacquemin E. Liver diseases related to MDR3 (ABCB4) gene deficiency. Front Biosci 2009; 14: 4242-56.
[113] Kreek MJ. Female sex steroids and cholestasis. Semin Liver Dis 1987; 7: 8-23.
[114] Reyes H, Simon FR. Intrahepatic cholestasis of pregnancy: an estrogen-related disease. Semin Liver Dis 1993; 13: 289-301.
[115] Laatikainen T, Karjalainen O. Excertion of progesterone metabolites in urine and bile of pregnant women with intrahepatic cholestasis. J Steroid Biochem 1973; 4: 641-8.
[116] Meng LJ, Reyes H, Palma J, Hernandez I, Ribalta J, Sjovall J. Profiles of bile acids and progesterone metabolites in the urine and serum of women with intrahepatic cholestasis of pregnancy. J Hepatol 1997; 27: 346-57.
[117] Reyes H, Sjovall J. Bile acids and progesterone metabolites in intrahepatic cholestasis of pregnancy. Ann Med 2000; 32: 94-106.
[118] Lindberg MC. Hepatobiliary complications of oral contraceptives. J Gen Intern Med 1992; 7: 199-209.
[119] Yamamoto Y, Moore R, Hess HA, *et al*. Estrogen receptor alpha mediates 17alpha-ethynylestradiol causing hepatotoxicity. J Biol Chem 2006; 281: 16625-31.
[120] Meyers M, Slikker W, Pascoe G, Vore M. Characterization of cholestasis induced by estradiol-17 beta-D-glucuronide in the rat. J Pharmacol Exp Ther 1980; 214: 87-93.
[121] Vallejo M, Briz O, Serrano MA, Monte MJ, Marin JJ. Potential role of trans-inhibition of the bile salt export pump by progesterone metabolites in the etiopathogenesis of intrahepatic cholestasis of pregnancy. J Hepatol 2005; 44: 1150-7.
[122] Huang L, Smit JW, Meijer DK, Vore M. Mrp2 is essential for estradiol-17beta(beta-D-glucuronide)-induced cholestasis in rats. Hepatology 2000; 32: 66-72.
[123] Akita H, Suzuki H, Ito K, *et al*. Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump. Biochim Biophys Acta 2001; 1511: 7-16.
[124] Takada T, Weiss HM, Kretz O, Gross G, Sugiyama Y. Hepatic transport of PKI166, an epidermal growth factor receptor kinase inhibitor of the pyrrolo-pyrimidine class, and its main metabolite, ACU154. Drug Metab Dispos 2004; 32: 1272-8.
[125] Mottino AD, Cao J, Veggi LM, Crocenzi F, Roma MG, Vore M. Altered localization and activity of canalicular Mrp2 in estradiol-17beta-D-glucuronide-induced cholestasis. Hepatology 2002; 35: 1409-19.
[126] Crocenzi FA, Mottino AD, Cao J, *et al*. Estradiol-17beta-D-glucuronide induces endocytic internalization of Bsep in rats. Am J Physiol 2003; 285: G449-59.
[127] Roma MG, Crocenzi FA, Mottino AD. Dynamic localization of hepatocellular transporters in health and disease. World J Gastroenterol 2008; 14: 6786-801.
[128] Crocenzi FA, Sanchez Pozzi EJ, Ruiz ML, *et al*. Ca(2+)-dependent protein kinase C isoforms are critical to estradiol 17beta-D-glucuronide-induced cholestasis in the rat. Hepatology 2008; 48: 1885-95.
[129] Fattinger K, Funk C, Pantze M, *et al*. The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions. Clin Pharmacol Ther 2001; 69: 223-31.
[130] Masubuchi Y. Metabolic and non-metabolic factors determining troglitazone hepatotoxicity: a review. Drug Metab Pharmacokinet 2006; 21: 347-56.
[131] Julie NL, Julie IM, Kende AI, Wilson GL. Mitochondrial dysfunction and delayed hepatotoxicity: another lesson from troglitazone. Diabetologia 2008; 51: 2108-16.
[132] Preininger K, Stingl H, Englisch R, *et al*. Acute troglitazone action in isolated perfused rat liver. Br J Pharmacol 1999; 126: 372-8.
[133] Funk C, Ponelle C, Scheuermann G, Pantze M. Cholestatic potential of troglitazone as a possible factor contributing to troglitazone-induced hepatotoxicity: *in vivo* and *in vitro* interaction at the canalicular bile salt export pump (Bsep) in the rat. Mol Pharmacol 2001; 59: 627-35.
[134] Stapelbroek JM, van Erpecum KJ, Klomp LW, Houwen RH. Liver disease associated with canalicular transport defects: current and future therapies. J Hepatol 2010; 52: 258-71.
[135] Davit-Spraul A, Fabre M, Branchereau S, *et al*. ATP8B1 and ABCB11 analysis in 62 children with normal gamma-glutamyl transferase progressive familial intrahepatic cholestasis (PFIC): Phenotypic differences between PFIC1 and PFIC2 and natural history. Hepatology 2010; 51: 1645-55.
[136] Paumgartner G, Beuers U. Ursodeoxycholic acid in cholestatic liver disease: mechanisms of action and therapeutic use revisited. Hepatology 2002; 36: 525-31.
[137] Beuers U, Bilzer M, Chittattu A, *et al*. Tauroursodeoxycholic acid inserts the apical conjugate export pump, Mrp2, into canalicular membranes and stimulates organic anion secretion by protein kinase C-dependent mechanisms in cholestatic rat liver. Hepatology 2001; 33: 1206-16.
[138] Dumont M, Jacquemin E, Erlinger S. Effect of ursodeoxycholic acid on the expression of the hepatocellular bile acid transproters (Ntcp and bsep) in rats with estrogen-induced cholestasis. J Ped Gastroenterol Nutr 2002; 35: 185-91.
[139] Marschall HU, Wagner M, Zollner G, *et al*. Complementary stimulation of hepatobiliary transport and detoxification systems by rifampicin and ursodeoxycholic acid in humans. Gastroenterology 2005; 129: 476-85.

[140] Dombrowski F, Stieger B, Beuers U. Tauroursodeoxycholic acid inserts the bile salt export pump into canalicular membranes of cholestatic rat liver. Lab Invest 2006; 86: 166-74.
[141] Fickert P, Wagner M, Marschall HU, *et al*. 24-norUrsodeoxycholic acid is superior to ursodeoxycholic acid in the treatment of sclerosing cholangitis in Mdr2 (Abcb4) knockout mice. Gastroenterology 2006; 130: 465-81.
[142] Kremer AE, Beuers U, Oude-Elferink RP, Pusl T. Pathogenesis and treatment of pruritus in cholestasis. Drugs 2008; 68: 2163-82.
[143] EASL Clinical Practice Guidelines: management of cholestatic liver diseases. J Hepatol 2009; 51: 237-67.
[144] Tandon P, Rowe BH, Vandermeer B, Bain VG. The efficacy and safety of bile Acid binding agents, opioid antagonists, or rifampin in the treatment of cholestasis-associated pruritus. Am J Gastroenterol 2007; 102: 1528-36.
[145] Makishima M, Okamoto AY, Repa JJ, *et al*. Identification of a nuclear receptor for bile acids. Science 1999; 284: 1362-5.
[146] Parks DJ, Blanchard SG, Bledsoe RK, *et al*. Bile acids: natural ligands for an orphan nuclear receptor. Science 1999; 284: 1365-8.
[147] Neimark E, Chen F, Li X, Shneider BL. Bile acid-induced negative feedback regulation of the human ileal bile acid transporter. Hepatology 2004; 40: 149-56.
[148] Pellicciari R, Fiorucci S, Camaioni E, *et al*. 6alpha-ethyl-chenodeoxycholic acid (6-ECDCA), a potent and selective FXR agonist endowed with anticholestatic activity. J Med Chem 2002; 45: 3569-72.
[149] Zollner G, Trauner M. Nuclear receptors as therapeutic targets in cholestatic liver diseases. Br J Pharmacol 2009; 156: 7-27.
[150] Liu Y, Binz J, Numerick MJ, *et al*. Hepatoprotection by the farnesoid X receptor agonist GW4064 in rat models of intra- and extrahepatic cholestasis. J Clin Invest 2003; 112: 1678-87.
[151] Dhawan A, Mitry RR, Hughes RD. Hepatocyte transplantation for liver-based metabolic disorders. J Inherit Metab Dis 2006; 29: 431-5.
[152] Waelzlein JH, Puppi J, Dhawan A. Hepatocyte transplantation for correction of inborn errors of metabolism. Curr Opin Nephrol Hypertens 2009; 18: 481-8.
[153] Meier Y, Pauli-Magnus C, Zanger UM, *et al*. Interindividual variability of canalicular ATP-binding-cassette (ABC)-transporter expression in human liver. Hepatology 2006; 44: 62-74.
[154] Hayashi H, Sugiyama Y. 4-phenylbutyrate enhances the cell surface expression and the transport capacity of wild-type and mutated bile salt export pumps. Hepatology 2007; 45: 1506-16.
[155] Feillet F, Leonard JV. Alternative pathway therapy for urea cycle disorders. J Inherit Metab Dis 1998; 21 Suppl 1: 101-11.
[156] Nagasaka H, Yorifuji T, Kobayashi K, *et al*. Favorable effect of 4-phenylacetate on liver functions attributable to enhanced bile salt export pump expression in ornithine transcarbamylase-deficient children. Mol Genet Metab 2010; 100: 123-8.

Received: January 23, 2010 Revised: March 27, 2010 Accepted: March 27, 2010

PMID: 21039337

ABCC6 as a Target in Pseudoxanthoma Elasticum

András Váradi*, Zalán Szabó, Viola Pomozi, Hugues de Boussac, Krisztina Fülöp and Tamás Arányi

Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary

Abstract: The *ABCC6* gene encodes an organic anion transporter protein, ABCC6/MRP6. Mutations in the gene cause a rare, recessive genetic disease, pseudoxanthoma elasticum, while the loss of one *ABCC6* allele is a genetic risk factor in coronary artery disease. We review here the information available on gene structure, evolution as well as the present knowledge on its transcriptional regulation. We give a detailed description of the characteristics of the protein, and analyze the relationship between the distributions of missense disease–causing mutations in the predicted three-dimensional structure of the transporter, which suggests functional importance of the domain-domain interactions. Though neither the physiological function of the protein nor its role in the pathobiology of the diseases are known, a current hypothesis that ABCC6 may be involved in the efflux of one form of Vitamin K from the liver is discussed. Finally, we analyze potential strategies how the gene can be targeted on the transcriptional level to increase protein expression in order to compensate for reduced activity. In addition, pharmacologic correction of trafficking-defect mutants or suppression of stop codon mutations as potential future therapeutic interventions are also reviewed.

Keywords: Genetic disease, connective tissue, cardiovascular, transcriptional regulation, calcification, vitamin K, membrane proteins, homology model.

PSEUDOXANTHOMA ELASTICUM

Pseudoxanthoma elasticum (PXE, OMIM 264800) is a recessive genetic disorder with a prevalence of 1 : 25.000 – 100.000, affecting the elastic tissues of the body, including the skin, the arteries and the elastic Bruch's membrane in the eye. Patients most commonly present with characteristic papules in the skin during late childhood or adolescence and subsequently develop angioid streaks of the retina. Angioid streaks are associated with subretinal neovascularisation, which can lead to hemorrhage and partial or complete loss of central vision. The diagnosis of pseudoxanthoma elasticum is suspected in individuals with characteristic skin and ocular findings and is confirmed by histological findings on biopsy of lesional skin in which fragmented calcified elastic fibers are visualized by use of special histological stains (e.g. von Kossa staining).

The disease was first described in 1881 by D. Rigal [1] and Félix Balzer, but the term *Pseudoxanthoma elasticum* (PXE) was first used in 1896 by Jean-Ferdinand Darier [2]. In 1889 Robert W. Doyne was the first to describe angioid streaks, than Ester Grönblad and James Strandberg revealed the connection between PXE and angioid streaks in 1929 [3].

CUTANEOUS AND MUCOSAL MANIFESTATIONS

The first sign of PXE is usually yellowish papules on the neck and other flexor surfaces. These skin lesions vary in size from 1-5 mm and may be grouped or coalesce to form larger plaques (Fig. **1A**). In most of the cases the skin loses its elasticity and becomes wrinkled and redundant. In addition to the neck, plaques may also appear on other areas, such as axillae, inguinal region, antecubital and popliteal fossae, and periumbilical area during the progression of the disease. In some cases mucosal lesions, identical to the skin lesions, can be detected on the inside of the lower lip, vagina, and all along the digestive tract mucosal membrane [4].

The classic histological findings in PXE are ultrastructural elastic tissue abnormalities in the middle and lower dermis. In PXE patients elastin becomes fragmented and degenerated (See Fig. **1C**). Deposition of calcium in the abnormal elastin matrix can be visualized by von Kossa or other histological stains for calcium [5]. Similar clinical findings may also be present is some other diseases for example in beta-thalassemia [6], focal dermal elastosis [7], cutis laxa [8], calciphylaxis [9] or Paget's disease [10].

However the ultra-structural histopathology is a hallmark of PXE. Even the skin findings in a highly related disease (PXE-like disorder with multiple coagulation factor deficiency) are in detail slightly different from the skin lesions found in PXE patients [11].

EYES

Symptoms eventually appear in the eyes in all cases of PXE (Fig. **1B**). The affected areas are the Bruch's membrane and the retinal-pigmented epithelium (RPE).

In PXE, calcification of dystrophic elastic fibers can be observed in the elastic layer of Bruch's membrane, similar to what is seen in the skin. Pigment irregularities, called peau d'orange, may appear in the RPE. Calcification and thickening of the Bruch's membrane, as well as loss of RPE pigment granules lead to the development of angioid streaks (AS) [12].

AS are dehiscences in Bruch's membrane, forming grayish to reddish irregular lines resembling vessels, emanat-

*Address correspondence to this author at the Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary; Tel: 3612733128; Fax: 3614665465; E-mail: varadi@enzim.hu

1389-4501/11 $58.00+.00 © 2011 Bentham Science Publishers Ltd.

ing from the optic disk. Due to the calcium deposition, Bruch's membrane becomes fragile, which is thought to be the major factor resulting in the dehiscences within the membrane. Later in the course of the disease fibrovascular tissue may grow through the damaged membrane, leading to choroidal neovascularization (CNV), subretinal fibrosis, atrophy of the overlying RPE and retinal hemorrhages [13].

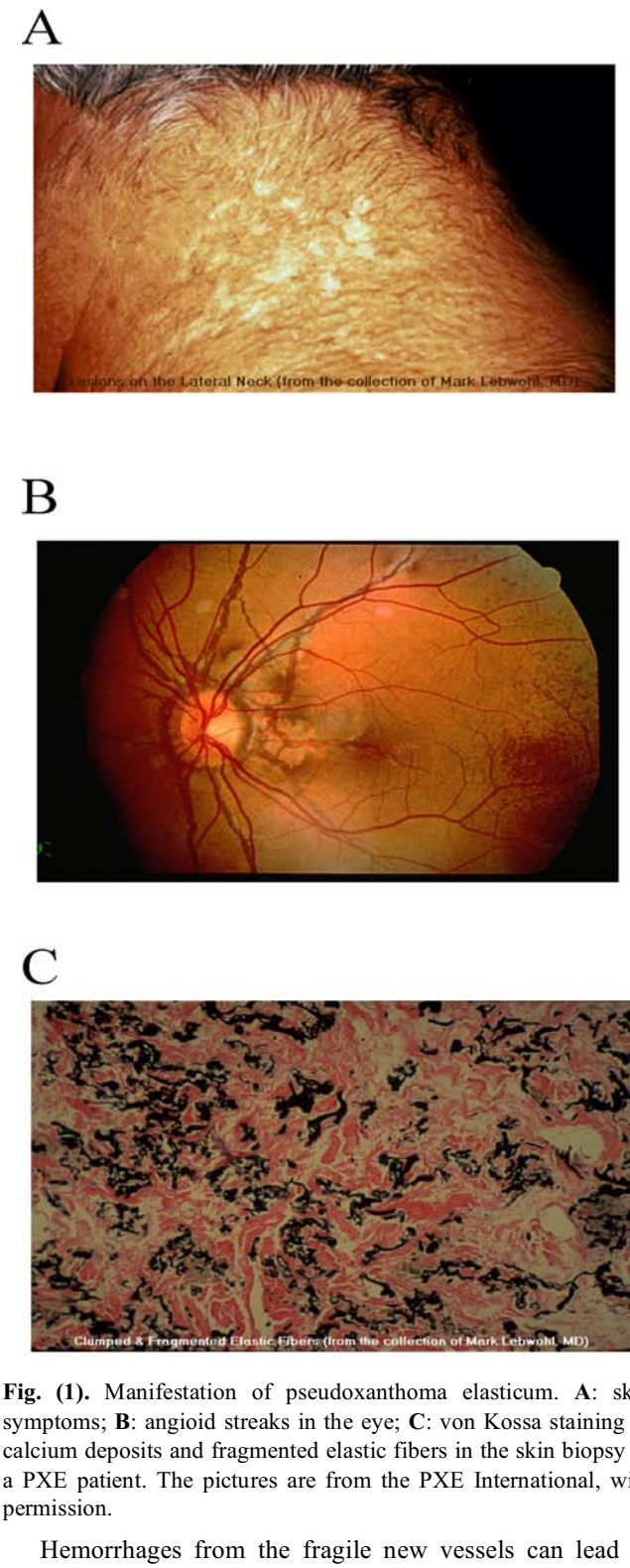


Fig. (1). Manifestation of pseudoxanthoma elasticum. **A**: skin symptoms; **B**: angioid streaks in the eye; **C**: von Kossa staining of calcium deposits and fragmented elastic fibers in the skin biopsy of a PXE patient. The pictures are from the PXE International, with permission.

Hemorrhages from the fragile new vessels can lead to partial or complete vision loss in PXE patients, starting usually around the third or fourth decade of life. Central vision loss has the greatest impact on the quality of life in PXE patients, but thus far no preventive measures are available. Laser therapy can be used to stop the proliferation or the bleeding of submacular neovessels, but it may cause visual loss or central scotomas due to scarring, and a high rate of recurrence has been observed [14, 15]. A promising therapy to treat ocular symptoms of PXE may be the application of antiangiogenic drugs. There have been several publications documenting efficacy of intravitreal treatment with vascular endothelial growth factor (VEGF) monoclonal antibodies in case of age-related macular degeneration resulting in CNVs [16, 17]. Similar treatment was already applied for a small group of PXE patients, showing very promising results [18].

CARDIOVASCULAR SYSTEM

Calcification may affect the cardiovascular system, mostly the small and middle-sized arteries. Cardiovascular symptoms include diminished peripheral pulses, angina pectoris, hypertension, mitral-valve prolapse and restrictive cardiomyopathy [19-23]. One of the most common cardiovascular symptoms is intermittent claudication. Bleeding, especially gastrointestinal hemorrhages may also occur probably due to calcification of the elastic fibers in the small arteries located under the mucosa [24]. Myocardial infarction or other symptoms leading to sudden death are rare, but probably the most serious complication associated with PXE is the early onset of atherosclerosis [4].

ABCC6 AS A POTENTIAL GENETIC RISK FACTOR IN CORONARY ARTERY DISEASE (CAD)

A strong correlation between a sequence variant of the *ABCC6* gene (c.3421C>T leading to the p.R1141X nonsense mutation) and CAD has been demonstrated in a Dutch cohort [25]. However, a surprisingly high frequency of the mutant allele was observed in the control population raising doubts about the validity of this unique study on the association of *ABCC6* mutation carrier status and CAD. An independent population genetic study on a large Hungarian cohort confirmed the findings of the previous report [26]. A significant association of carrier status and CAD was observed (5/361 carriers p=0.016 OR=10.5 95%CI 1.22-90.30). These findings mean that one non-functional allele of *ABCC6* increases the risk of CAD significantly.

THE *ABCC6* GENE

In 2000 it was discovered by positional cloning that mutations in the *ABCC6* gene are responsible for the development of PXE [27-29]. Since the discovery of the connection between *ABCC6* mutations and PXE a large number of disease-causing mutations has been identified; the most frequent ones are p.R1141X (20 – 30%) and c.EX23_29del (5-15%). The high heterogeneity of PXE alleles in the population is comparable to that of other autosomal diseases. A locus-specific database has been established recently with the collection of the disease-causing mutations and other genetic variants and with link to other genetic databases [www.ncbi.nlm.nih.gov/lovd/home.php?select_db=ABCC6].

ABCC6 is located at 16p13.11 and codes for the ATP-binding cassette transporter protein, ABCC6/MRP6. The functional gene of 75 kb size consists of 31 exons. Two pseudogenes that are expressed at low levels and are positioned centromeric (*ABCC6-ψ1*) and telomeric (*ABCC6-ψ2*) of *ABCC6* have also been mapped [30]. Both *ABCC6-ψ1* and *ABCC6-ψ2* share a high degree of sequence similarity (~99%) with the functional gene, but are truncated in the fourth and ninth intron, respectively. The *ABCC6* locus is located in a genomic region that was subject to segmental duplications, a series of events thought to play a crucial role in the recent evolution of the *ABCC6* gene cluster [31]. Due to this evolutionary scenario chromosomal rearrangements, gene conversion and emergence of new genes have been observed.

There are several reports indicating that the *ABCC6* locus is genetically unstable. A rare fragile site (FRA16A) has been found in close centromeric proximity to *ABCC6* [32], while the breakpoint of rearranged Chromosome 16 in the acute non-lymphocytic leukemia cell line M4Eo was localized ~0.5 Mb telomeric of *ABCC6* [33, 34]. *ABCC1*, the gene located closest to *ABCC6* (8 kb apart) is frequently deleted in the drug-selected M4Eo cell line, and the concomitant amplification of *ABCC1* and *ABCC6* is observed in the SKOV3 ovarian carcinoma cell lines after multidrug selection [35].

TRANSCRIPTIONAL REGULATION

The initial characterization of the transcriptional regulation of the human *ABCC6* gene identified two evolutionarily conserved regions in the 5' sequence 10kb upstream from the translation start site [36]. Both regions harbor a CpG island (CGI), potential target of DNA methylation. Analysis of DNA methylation may give clues to the location of important regulatory regions of gene expression, as methylation is stable like an imprint. Methylated regions indicate silenced and unmethylated regions designate transcriptionally active sequences [37]. Bisulfite genomic sequencing was carried out to analyze both the distal and the proximal CGI in different cell lines expressing and non-expressing *ABCC6* [36]. Cell-type specific DNA methylation in the proximal CpG island was detected, which inversely correlated with the expression of the gene and suggested that this region plays an important role in the tissue-specific regulation of *ABCC6*.

Based on these data luciferase reporter gene assays were performed with sequential deletion promoter constructs. One silencer (between -713 and -332 bp) and one DNA methylation sensitive activator sequence (between -332 and -145 bp) were identified [36]. These data indicated that this region confers tissue-specificity to the *ABCC6* expression pattern. Further promoter mapping experiments confirmed these findings by identifying one tissue-specific regulator element (between -209 and -145 bp) and one further stronger activator sequence located between -234 and -209 bp [38].

The potential regulatory role of some transcription factors and cytokines has been suggested. The binding of the PLAG family of transcription factors and RXR has been convincingly demonstrated: they are able to transactivate the endogenous *ABCC6* gene, the binding site was determined by luciferase assay and the binding to the *ABCC6* promoter in the natural chromatin environment was demonstrated by chromatin immunoprecipitation. However, their functional role is still unclear [38, 39]. The binding of NF-κB, SP1 and TGF-β has been also suggested but their functional role and their binding to the endogenous *ABCC6* promoter have not been tested [40].

Signal transduction pathways leading to the modulation of *ABCC6* expression have also been deciphered. Initially the activation by TGF-β and inhibition by TNF-α and IFN-γ were reported in luciferase reporter gene assays [40]. However, these effects have not yet been confirmed on the endogenous gene and the implicated signal transduction pathway was not identified. More recently, we found that activation of the MAP kinase ERK1/2 cascade leads to the significant inhibition of the expression of *ABCC6* in HepG2 and Caco-2 cell lines [41]. The detailed analysis of the molecular mechanism of this inhibition of ABCC6 expression revealed that this is a direct effect on transcription initiation. Indeed, it was found that a degenerate but functional HNF4 (hepatocyte nuclear factor 4) binding site [42, 43] plays a pivotal role in the regulation of ABCC6 [41]. While HNF4 binds tightly to the promoter of the gene under normal conditions this occupancy is dramatically reduced upon the activation of the ERK1/2 pathway leading to the decreased expression of ABCC6.

These experiments also revealed that mutated ABCC6 promoter constructs with abolished HNF4 binding site have prevented further activation of the luciferase activity by the other transcription factors [41]. This strongly suggests that HNF4 is responsible for the tissue-specific regulation of ABCC6. It is worth to note that ABCC6 is expressed at a detectable level only in tissues where HNF4 is expressed [44, 45].

Our further experiments on the transcriptional regulation of ABCC6 identified a strong primate- and tissue- specific enhancer located in the first intron of the gene. We also demonstrated that the proteins binding to this enhancer form an activator complex with the proteins binding to the proximal promoter [46].

MODIFYING GENES AND PXE PHENOCOPIES

The PXE phenotype is highly variable even within a single family where patients have the same disease-causing mutations [4]. No clear genotype/phenotype correlation has been observed to date [47]. Finally, other diseases can mimic the PXE phenotype. The PXE-like syndrome is due to mutations in the gamma-glutamyl carboxylase (*GGCX*) gene [11], while certain genetic hemoglobinopathies (e.g. thalassemia) lead to slowly developing phenotypes similar to PXE [48]. Although the molecular mechanisms of the developing phenotype are not yet understood, in beta thalassemia mice a liver-specific down-regulation of *Abcc6* gene expression was observed [49]. Christian Gotting and his coworkers identified a number of genes modifying the disease course in a German cohort. They demonstrated that the *ABCC6* c.-219A>C promoter polymorphism is significantly less frequent in patients than in the control population [50]. They also showed that certain promoter polymorphism of the *SPP1* (secreted phosphoprotein 1, previously called: osteopontin) gene were more frequent in PXE patients than in

controls [51]. Furthermore, earlier disease onset is associated with polymorphisms of catalase, superoxide dismutase and glutathione peroxidase genes [52]. They also found that polymorphisms of the *VEGF* (vascular endothelial growth factor gene) are prognostic markers for ocular symptoms [53]. Similarly, it was observed that the c.2402C>G p.T801R polymorphism of the xylol-transferase II gene is associated with increased PXE severity [54].

THE ABCC6/MRP6 PROTEIN

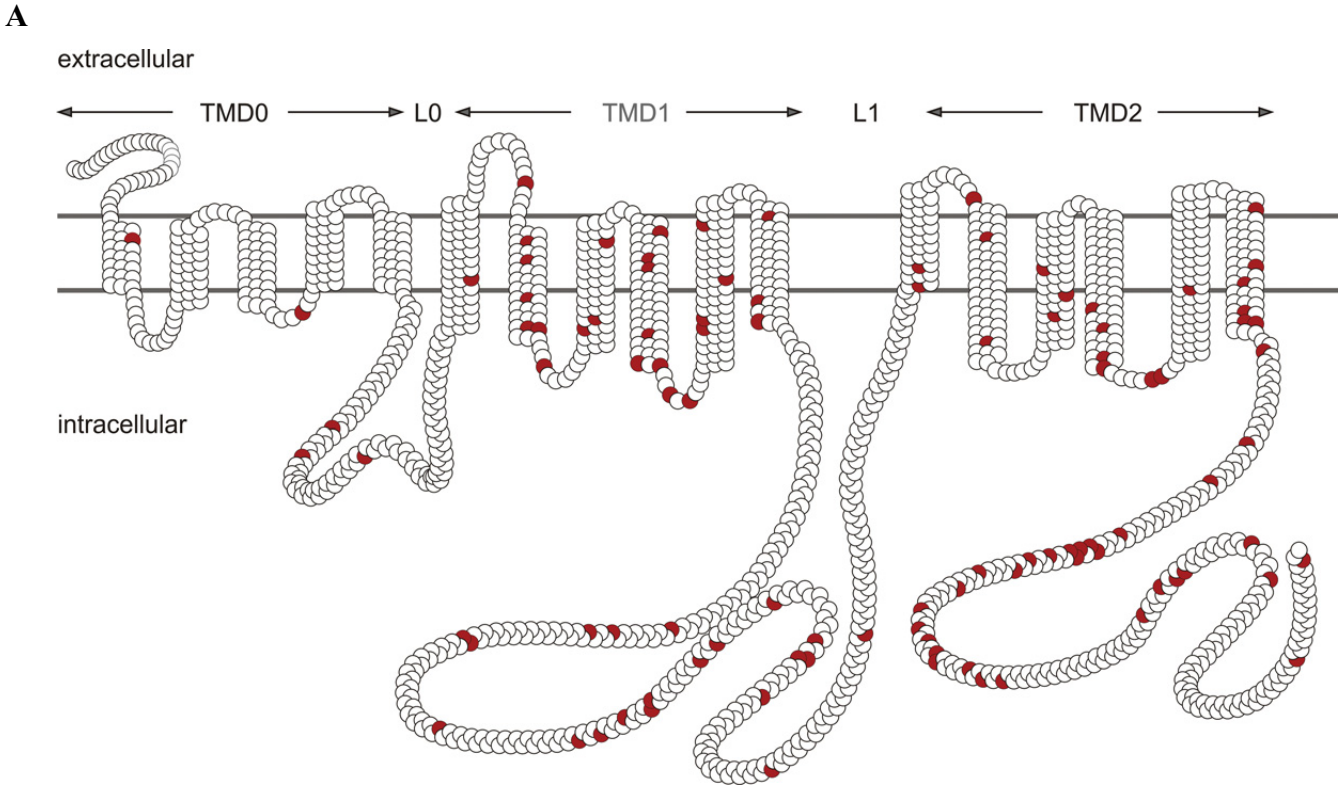
The human proteome contains 48 ABC proteins; on the basis of sequence similarity they are grouped into seven subfamilies from A to G. The ABCC-subfamily includes twelve members; most of them are active transporters while ABCC7 (CFTR) acts as a chloride channel and probably regulates the action of other ion channels *(for more details, see the relevant chapter of the present issue: CFTR [ABCC7] target in Cystic Fibrosis).* Two other members of the subfamily, ABCC8 and 9 are K^+-channel regulators operating as intracellular ATP/ADP sensors thus reporting about the metabolic state of the cells *(for more details, see the relevant chapter of the present issue: ABCC8/9 target in type 2 diabetes).*

The ABCC-proteins share the general features of the ABC-kingdom: they harbor two nucleotide-binding (ABC)-domains and two transmembrane domains (TMDs), each with six membrane-spanning helices (the so called "core structure"). It is a unique feature of some ABCC-type proteins ("long MRPs" like ABCC1, 2, 3, 6, 8, 9 and 10) that two additional domains are attached to the core structure N-terminally: a transmembrane domain with five membrane spanning helices and an intracellular loop. Accordingly, the domain architecture of the long MRPs, including ABCC6/MRP6 is TMD0-L0-TMD1-ABC1-L1-TMD2-ABC2 (L0 and L1 are intracellular loops) (Fig. **2** panel **A**). ABCC6 consists of 1503 amino acids and it is known that the protein functions as an organic anion transporter [55, 56]. Indeed, *in vitro* studies demonstrated the transport of glutathione-conjugates like glutathione S-conjugated leukotriene C4 (LTC4), N-ethylmaleimide S-glutathione (NEM-GS) and S-(2,4-dinitrophenyl) glutathione, while the rat orthologue transports an anionic cyclopentapeptide [57]. It has also been shown by *in vitro* assays that some missense mutations described as causative mutations in pseudoxanthoma elasticum result in the loss of ATP-dependent transport of test substrates [55]. Compared with its sub-family members (ABCC1-5) ABCC6 is a poorly characterized transporter. The protein shows significantly lower transport rate (turnover number) in *in vitro* assays than the other human ABCC-type transporters, which makes its detailed biochemical/functional characterization difficult.

It has been suggested that overexpression of ABCC6 is able to confer low level of resistance to several commonly used natural product anticancer agents like etoposide, doxorubicin, daunorubicin and actinomycin D [56]. However, clinically relevant ABCC6-mediated drug resistance has never been found.

Homology Models

No high-resolution three dimensional structure of ABCC6 is available. However, a three dimensional homo-



(Fig. 2) Contd…..

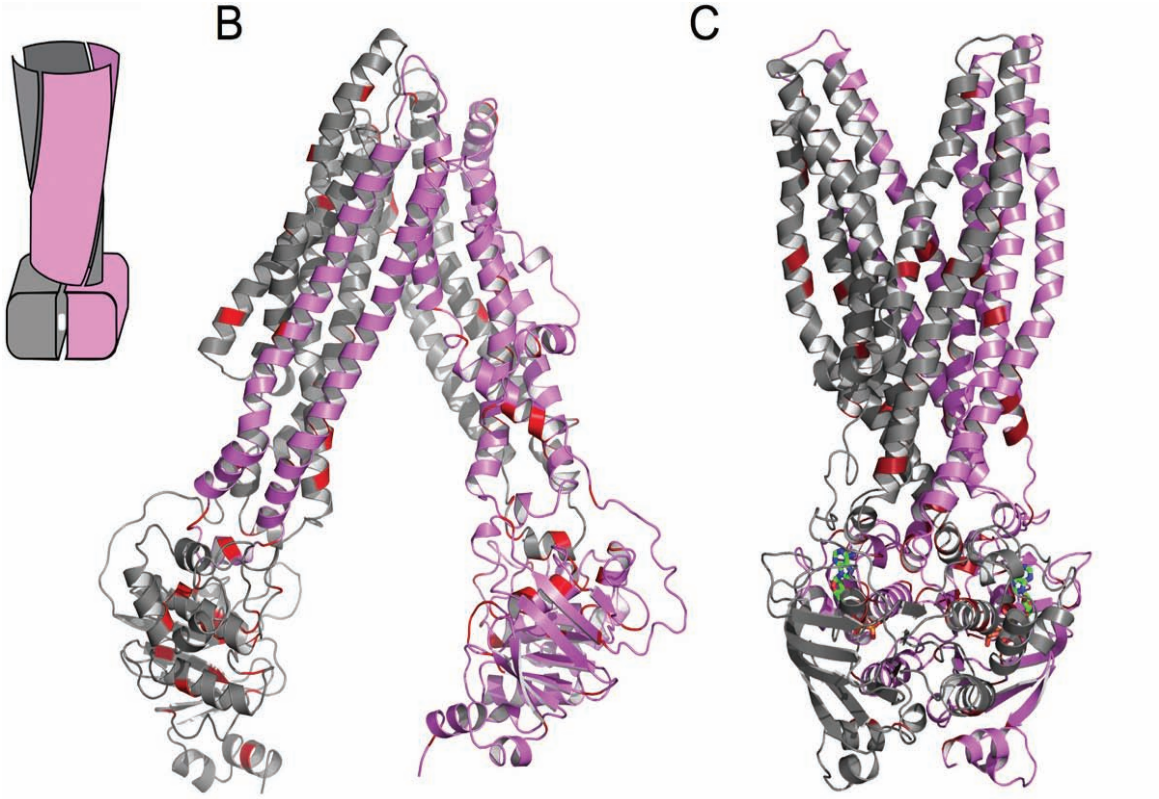


Fig. (2). Membrane topology and three dimensional homology models of ABCC6. Missense mutations are indicated in red. **A**: the membrane homology model and domain arrangements of ABCC6. **B**: three dimensional homology model of ABCC6 representing the outward facing conformation; **C**: three dimensional homology model of ABCC6 representing the nucleotide-free conformation, Insert: schematic representation of the domain swapping of ABC proteins.

logy model of ABCC6 is already built and published [58], made possible by the recent publication of high resolution crystalline structures of ABC proteins [59-61]. One of the structures representing the nucleotide-saturated, outward facing conformation shows that the two nucleotide-binding (ABC) domains are in close proximity to each other in the characteristic head-to-tail orientation reflecting to the previously described "nucleotide sandwich dimer" [62]. The other shows a nucleotide-free, substrate-saturated conformation. Newly recognized structural elements are the long "rigid" extensions of the transmembrane helices, called intracellular loops (ICL). Each half of the ABC proteins has two ICLs interacting with the ABC-domains. The coupling helices contact with their “own” as well as with the “opposite” ABC-domains, hence a special type of domain swapping can be recognized in the structure (see insert on Fig. **2**).

We have constructed two homology models of human ABCC6 protein: one of the models is based on the Sav1866 bacterial ABC transporter structure [58] representing a nucleotide-saturated conformational state, while the other one uses the recently published mouse Abcb1 structure as template and represents the nucleotide-free (apo) conformation. The two models are illustrated on Fig. (**2**), Panel **B** and **C**. By performing a statistical analysis we have found a significant clustering of the missense PXE-mutations at the domain-domain interfaces: at the transmission interface that involves four intracellular loops (ICLs) and the two ABC domains as well as at the ABC - ABC interacting surfaces. In the nucleotide-saturated model the mutations affecting these regions are 2.75 and 3.53 fold more frequent than the average mutational rate along the protein sequence, respectively [58]. At the predicted ICL-ABC interfaces in the nucleotide-free model the mutational rate is 4.25-fold more frequent than the average mutational rate along the protein sequence (the ABC domains are distant in this conformation). The observed significant clustering means that the domain contacts are much less permissive to amino acid replacements than the rest of the protein. These results provide a “bridge” between genetic data and protein structure and can be viewed as novel proof of the importance of the studied domain-domain interactions in the ABCC6 transporter.

ANIMAL MODELS

Abcc6 knock out mouse models were generated and the critical role of *Abcc6* in ectopic mineralization/calcification has been confirmed in the *Abcc6*$^{-/-}$ mice which recapitulates the genetic, histopathologic and ultrastructural features of PXE [63, 64]. These findings suggest that the function of this transporter is conserved in the mouse. Calcification in the vibrissae capsules is the first symptom of the calcification phenotype detected at the 8 to 10 weeks of age and serves as an early biological marker of the disease [63]. A slight

alteration of plasma lipid composition of the *Abcc6*$^{-/-}$ mice has also been reported [64]. The KO mouse models have been utilized for physiological and for pharmacological studies that are discussed elsewhere in this paper.

Dystrophic Cardiac Calcification (DCC) in the mouse is an autosomal recessive trait in certain laboratory strains and the *Abcc6* gene locus has been recently found as a main mediator of DCC at the Dyscalc1 locus [65, 66]. A splicing error in processing of *Abcc6* mRNA has been identified as the causative genetic event ("splice-mutation") of DCC. This mouse shows a more pronounced arterial calcification phenotype than the one observed in the laboratory-generated *Abcc6*$^{-/-}$ mice strains (presumably due to the different genetic background) and seems to be as good model of PXE as the latter.

The zebrafish (Danio rerio) has nearly the same *ABC* gene repertoire as the human and has accessible and well-characterized embryo. Two morpholinos were designed targeting two different regions of the *Abcc6a* gene, and it was observed that they decrease *Abcc6a* expression by 54 and 81%. Both morpholinos induced a similar phenotype, cardiac edema and curled tail. Microinjecting zebrafish larvae with full-length mouse *Abcc6* mRNA completely rescued the knockdown phenotype [67]. These recent results serve as basis of a novel knockdown animal model system. However, the results provided by this model may not be translated directly to human physiology, as the zebrafish gene appears to be essential for the development of the animal.

THE "VITAMIN K HYPOTHESIS" OF PXE

ABCC6 is predominantly expressed in the liver in the basolateral compartment of the plasma membrane of the hepatocyte (and to a lesser extent in the kidney and the intestine), while the symptoms are systemic affecting various organs. This apparent discrepancy led to the hypothesis that PXE is a metabolic disease suggesting that ABCC6 is involved in the secretion of a metabolite from the liver into the circulation [68]. Recent experiments demonstrated that grafting of wt mouse muzzle skin onto the back of KO mice triggered mineralization, whereas grafting KO mouse muzzle skin onto wt mice was accompanied with no mineralization [69]. These transplantation experiments argue that PXE is indeed a metabolic disorder. Furthermore, in a parabiotic experiment the surgical pairing of Abcc6(-/-) mice with wild-type prevented the mineralization of the connective tissue in the knockout mice [70].

There have been a few case reports of a disease that phenotypically resembled pseudoxanthoma elasticum with respect to the mineralization of soft tissues causing cardiovascular, dermal and ocular symptoms. However, these patients suffer from a vitamin K-dependent coagulation factor deficiency which is not seen in PXE [71-73]. The disorder is extremely rare and for decades its molecular basis remained unknown. Also, no mutations in the *ABCC6* gene could be detected in these individuals, suggesting that mutation of another gene could also cause PXE-like soft tissue calcification. The identity of this enigmatic gene was unraveled recently [11], and the clinical condition was classified as a novel disorder: PXE-like disease (pseudoxanthoma elasticum-like disorder with multiple coagulation factor deficiency, OMIM 610842). Six patients were found to possess compound heterozygous mutations in the gamma-glutamyl carboxylase (*GGCX*) gene.

The *GGCX* gene encodes the gamma-glutamyl carboxylase enzyme (GGCX), an ER (endoplasmic reticulum)-resident protein, responsible for post-synthetic carboxylation of Gla-domain containing proteins to which they confer Ca-binding properties [74]. During the carboxylation reaction, vitamin K (VitK) is oxidized to an epoxide form, which is then re-reduced by another enzyme, Vitamin K oxidoreductase (VKORC1), thereby completing the VitK-cycle [75]. Proteins with Gla residues bind calcium (and certain other divalent cations) and this property is required for their physiological function. The best-known members of this group of proteins are the vitamin K-dependent coagulation factors produced in and secreted from the liver. The reduced activity of the GGCX enzyme explains the coagulation deficiency in PXE-like patients. Another gamma glutamyl carboxylated protein is MGP (matrix gla protein) which is a potent inhibitor of connective tissue mineralization and its function is essentially dependent on the correct carboxylation of the protein [76-78]. Insufficient carboxylation of MGP might thus be responsible for the soft tissue calcification in PXE-like patients. MGP can be detected in mineralized tissues of individuals with classic PXE as well as in *Abcc6* mutant mice [79-81]. Using antibodies specifically recognizing the non-carboxylated and the carboxylated forms of MGP, it has been shown that sites of ectopic calcification only contained the undercarboxylated form of MGP [80, 81]. These data led to the hypothesis that ABCC6 could directly or indirectly influence the availability of vitamin K, or the capacity of the vitamin K cycle at peripheral tissues. The highly similar phenotypic features of the two diseases evoked hypotheses about their overlapping pathophysiology. In PXE-like disease – due to the mutations of the GGCX enzyme - the Gla-gammacarboxylation is reduced in the liver, thus resulting in blood coagulation abnormality, and also in the extrahepatic soft tissues where the control of calcification is impaired. In classical PXE gamma carboxylation is normal in the liver as there is no mutation in *GGCX*. According to the current hypothesis, in extrahepatic tissues gamma carboxylation is lower than normal as Vitamin K available for the carboxylation cycle may be limited in those tissues.

This notion is supported by the very recent finding that the level of circulating vitamin K1 is lower in PXE patients as compared to healthy controls [82]. It is notable that the variability between patients is high and the range of measured vitamin K1 levels in the control group overlaps with that of the PXE patient group. Collectively, these data raises the possibility that one form of Vitamin K is transported from the liver into the circulation, and this transport is mediated by ABCC6 (and is missing in PXE due to ABCC6 mutations) [83].

On the other hand, the fact that PXE is a slowly progressive disease suggests that the metabolite transported by ABCC6 may only be reduced but not completely absent in the circulation of patients. Besides being a co-factor of gamma carboxylation, VitK may have other physiological functions, including transcriptional regulation and protection

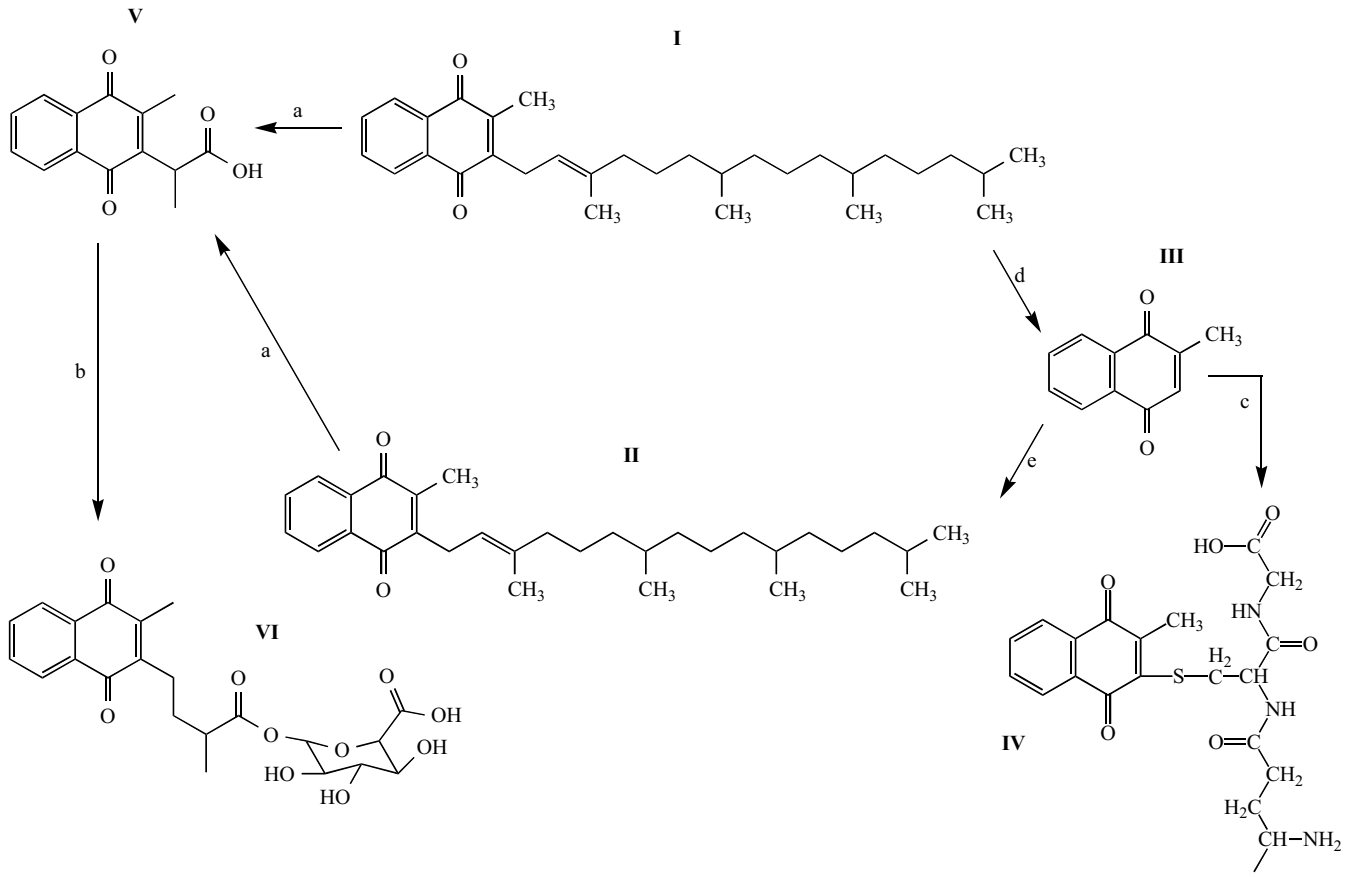


Fig. (3). The major Vitamin K forms and metabolites. **I**: VitK1; **II**. MK4 (VitK2); **III**: VitK3 (menadione); **IV**: VitK3-glutathione conjugate; **V**: VitK aglycone; **VI**: aglycone glucoronide. Beta-oxidation is represented by reaction **a**; glucuronidation by **b**; glutathione conjugation by **c**; conversion of VitK1 to VitK3 by **d**; while resynthesis of the sidechain generating MK4 by **e**.

of certain neuronal cells from oxidative injury [84]. These findings need to be taken into consideration when studying the connections between vitamin K status, ABCC6 and PXE. Whether there is a connection between protection against oxidative stress by VitK and the chronic oxidative stress measured in the serum and cells of PXE patients [52, 85, 86] is unknown and needs further investigation.

Currently, the exact metabolic pathway of Vitamin K is not known in detail. Dietary Vitamin K (phylloquinone, VitK1) is mostly utilized in the liver to serve as a cofactor in blood clotting factor synthesis. Part of Vitamin K1 is converted by side-chain removal to Vitamin K3 (also known as menadione) probably in the enterocytes [87]. K3 can be taken up by the extrahepatic tissues and the complex aliphatic side chain is substituted to the naphtoquinone core thus generating MK4 (a menaquinone, also called Vitamin K2), which is available for the Vitamin K cycle of the extrahepatic tissues [88]. It is known that both Vitamin K1 and MK4 are metabolized to a common catabolite after beta-oxidation of the side-chains and subsequent glucuronidation and the conjugate is secreted into the urine [89]. K3 can be conjugated with glutathione. The known metabolic events and vitamin K compounds are shown in Fig. (**3**).

In principle, any of the Vitamin K metabolites or molecular forms (see Fig. **3**) could be the transported substrate(s) of ABCC6, that, according to the "Vitamin K hypothesis", control(s) indirectly - via gamma-carboxylation of Ca-binding proteins like MGP - the formation of calcium-deposits in the arterial wall and in other soft tissues. However, the key role of Vitamin K in the disease phenotypes associated with ABCC6 has not been proven.

Indeed, very recent independent studies challenged the "Vitamin K theory". In one of the papers [90] it was demonstrated that oral administration of massive amount of vitamin K2 did not alter the ectopic mineralization in *Abcc6*$^{-/-}$ mice. Similarly, intravenous administration of Vitamin K3-glutathion conjugate (K3-GSH) did not alter the degree of mineralization. Furthermore, the same authors also found that vitamin K2, K3 and K3-GSH has no effect in an *in vitro* calcification system, i.e. they did not trigger any mineralization inhibition.

In the other study [91] *Abcc6*$^{-/-}$ mice were placed on a diet of either 5 or 100 mg/kg of vitamin K1 or K2 at prenatal, 3 weeks or 3 months of age. These authors also found no significant change in the levels of pathologic calcification irrespective which type of administration was used. However, measuring the plasma levels of different Vitamin K forms resulted in a very interesting observation: upon the same administration the level of Vitamin K1 in the plasma of wt mice was significantly higher than in the plasma of the Abcc6$^{-/-}$ animals. The same was true when Vitamin K2 was administrated orally. These results suggest

that Abcc6 might be involved in vitamin K transport, absorption or metabolism in the body to some degree, which is in agreement with the observations discussed in the previous paragraphs [81].

Unequivocally, both studies provided evidence that dietary supplementation in vitamin K is not a viable approach to prevent PXE-related calcification thus suggesting that the availability of vitamin K is not a limiting factor in the pathology of PXE.

Local expression of ABCC6 has been shown in several tissues and cell types, e.g. keratinocytes, fibroblasts, smooth muscle cells and macrophages [92-94], some of those are affected in PXE. The local effect of missing ABCC6 activity may also contribute to the progression of the disease.

ABCC6 AS A DRUG TARGET

Two disease conditions are associated with mutations in the *ABCC6* gene: pseudoxanthoma elasticum is a recessive trait due to mutations in both *ABCC6* alleles, while the loss of one functional *ABCC6* allele is a genetic risk factor in coronary artery disease, CAD and possibly stroke [95]. As the phenotype in both cases is due to complete or partial loss of ABCC6 activity, augmentation-type gene therapy – in principle – could be an effective treatment of the disease conditions. However, even if all the safety concerns of gene therapy were solved, there would be questions to be answered: is it sufficient to restore ABCC6 activity only in the liver, or it is also needed to do in other organs with lower level of expression (e.g. kidney)?

Some of the PXE-causing *ABCC6* mutations may result in only partial loss of function of the protein. Induction of expression of such a low activity mutant – in theory – could potentially be therapeutic in these cases. The induction of ABCC6 expression could be achieved at distinct levels: increased transcription, increased RNA stability or increased protein stability. However, we have almost no information to date about the regulation of ABCC6 RNA and protein stability. Therefore, currently the only way to develop a hypothesis-driven therapy based on increased ABCC6 expression is to intervene at the transcriptional regulation of the gene. As discussed previously the transcriptional regulation of *ABCC6* was investigated by different groups and the potential role of several transcription factors has been suggested [38-40]. However, only the role of ERK1/2-HNF4 pathway has been analyzed in a potential physiological context. As previously mentioned, HNF4 is a *bona fide* activator of the gene, while the activation of the ERK1/2 pathway inhibits HNF4 and thereby the expression of *ABCC6* [41].

ERK1/2 regulate several physiological processes, such as cell growth and proliferation, differentiation, survival and apoptosis [96]. The pathway is activated by a number of stimuli, like growth factors and environmental stresses (e.g. oxidative stress) or through various G protein coupled receptors [97]. The pathway has a low basal activity and upon activation it is rapidly deactivated by different phosphatases the most important among them being PP2A (protein phosphatase 2A). PP2A itself is under the control of protein kinase A and other signaling cascades [98].

An option to increase the expression level of the *ABCC6* gene would be the inhibition of ERK1/2 activation in hepatocytes. Due to the myriad of ERK1/2 activating factors, the low basal activity and the fast turnover of the signals, inhibitory stimuli of ERK1/2 are not easy to find. However, some of the anticancer therapies are targeting the ERK1/2 pathway and promising results have been obtained both *in vivo* and *in vitro* in inhibiting cancer progression [99, 100]. It is highly probable that new generations of these molecules will become available. These molecules might have beneficial effect in some patients because only a slight inhibitory effect would be necessary in the case of PXE symptoms, the toxic effect of the molecules on other tissues would be diminished. Furthermore, in some cases patients are suffering from chronic oxidative stress and develop secondary PXE (e.g. in beta-thalassemia) [48, 49]. These patients treated with ERK1/2 inhibitors and/or an anti-oxidative stress therapy might also receive therapeutic benefit. Similarly, the *ABCC6* mutation carriers also suffer from mild oxidative stress [52, 101], a condition potentially inhibiting the expression of the gene from the remaining allele. These patients could presumably benefit from the same therapy to prevent the development of CAD.

ERK1/2 has a wide variety of targets. One of them is HNF4, which is inactivated either directly or indirectly by the kinase [102, 103]. HNF4 transcription factor is a major regulator of the expression of *ABCC6* and also a master regulator of metabolism in hepatocytes. By influencing the global metabolic state of the liver one can induce or inhibit the expression and/or the activity of HNF4. Accordingly, it has been demonstrated that high insulin levels (e.g. in diabetic mouse models HNF4) is downregulated [104], while in fasting states functional HNF4 level is increased [105] leading to hepatic gluconeogenesis. Similarly, it has been shown, that HNF4 is up-regulated by glucocorticoid hormones [106]. These conditions might contribute to a higher *ABCC6* expression level and diminishing or eliminating the PXE symptoms in some PXE and probably the beta thalassemic patients.

Missense disease-causing mutations can reduce the transport activity and/or the overall stability of the transporter, or may result in a slightly altered conformation that is not compatible with the normal trafficking of the protein to the plasma membrane. Indeed, defective protein trafficking caused by mutations underlies many human diseases and examples include several membrane-embedded ABC-proteins (like ABCC7/CFTR, ABCC2, ABCC8/SUR1 or ABCB11/BSEP). Efforts to identify pharmacologic compounds to correct the misfolding and/or misprocessing of mutant membrane proteins have already resulted in a few remarkable findings, and are considered as the molecular basis of allele-specific therapy of the given disease (see e.g. [107]). This observation raises the possibility that pharmacological compounds (acting either as "chemical chaperones" or interfering with the quality control of the protein sorting/processing mechanism) may correct the defect causing the disease in a group of patients. Substrates and modulators of ABCB1 have been demonstrated to act as chemical chaperones thus helping the appearance of fully mature protein at the cell surface in the case of processing ABCB1 mutants [108]. The first effort of promising correction of folding of

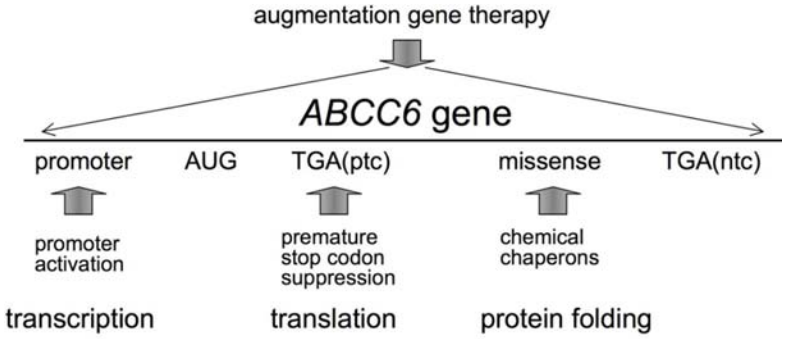


Fig. (4). Targets of potential therapeutical interventions. Solid vertical arrows represent the target of intervention; TGA(ptc) means TGA premature termination codon (e.g. R1141X in several PXE patients); TGA(ntc) means TGA at the natural termination codon position (1504 in ABCC6 mRNA).

the Δ508CFTR mutant was achieved by curcumin, which might act as a chemical chaperone [109].

Several studies demonstrated that sodium 4-phenyl-butyrate (4-PBA) can restore the cellular trafficking of a mutated ABCC7/CFTR (ΔF508) and its cellular function were restored – at least partly - in both cultured cells and in cystic fibrosis patients [110-112]. 4-PBA is approved for clinical use in human subjects in urea cycle disorder [113]. As a "chemical chaperon" it is thought to interfere with the Hsc70 protein in the endoplasmic reticulum, allowing a proportion of misfolded proteins to escape association with the chaperon thus improving cellular trafficking [114], and this effect is independent of its function as a drug of urea cycle disorders.

We have embarked upon a similar project on missense disease-causing mutants of ABCC6 utilizing *in vitro* and *in vivo* approaches [115]. First we study missense mutants by expression in insect cells. This establishes the transport characteristics and identifies those ABCC6 mutants with stability and preserved transport activity. To investigate the intracellular targeting of the ABCC6 mutants we developed a novel experimental approach to express the human ABCC6 variants in the liver of living mice. This approach provides a unique insight into the intracellular processing of a human ABC transporter in physiological conditions very similar to the biology of the fully differentiated human liver cells. Our preliminary results demonstrate that this complex experimental strategy is capable to determine those mutants which are potential targets of chemical chaperon- based allele-specific intervention. Indeed, by 4-PBA treatment of mice transiently expressing human ABCC6 variants we could identify one PXE mutant protein reverting its exclusively intracellular localization to a near normal plasma membrane distribution in mouse liver cells *in vivo*.

The most frequent mutation in PXE is the R1141X nonsense mutation. It has been shown that aminoglycoside antibiotics like gentamicin can suppress premature stop codon arrest of translation by inducing the ribosome to read through (or "suppress") the nonsense mutation via insertion of an amino acid. It was demonstrated in a muscular dystrophy mouse model that aminoglycosides could suppress stop codons not only *in vitro* but also *in vivo* [116]. However, the efficacy of the "read through" may be quite low. Enhanced production of active protein from genes with nonsense mutations can be achieved by combining treatment by inducing the promoter of the gene and the application of "read through" agents [117]. A promising new non-aminoglycoside agent of suppressing premature termination codons is PTC124 (Ataluren), which is in clinical trial in cystic fibrosis, in Hemophilia A and B and in Duchenne muscular dystrophy [118]. These strategies can now be tested in experiments with the aim of correcting the frequent ABCC6 R1141X stop codon mutation.

In Fig. (**4**) we have summarized the potential targets of the therapeutical interventions discussed above.

ACKNOWLEDGEMENT

This work has been supported by Hungarian research grants OTKA NI 68950, NHTK-OTKA CK 80135, OTKA PD 79183, KMOP, Bolyai János fellowship (to TA) and by NIH RO1 AR055225 (to AV). Images on Fig. (**1**) were kindly provided by PXE International, while the help of László Barna (Institute of Enyzmology) in creating Fig. (**3**) is greatly appreciated.

ABBREVIATIONS

ABC	=	ATP-binding cassette
ABCC6	=	ATP-binding cassette protein, family C, number 6
AS	=	Angioid streak
CAD	=	Coronary artery disease
CGI	=	CpG island
ChIP	=	Chromatin immunoprecipitation
CNV	=	Choroidal neovascularization
DCC	=	Dystrophic cardiac calcification
ERK1/2	=	Extracellular signal-regulated kinase 1/2
GGCX	=	γ-Glutamyl carboxylase
HNF4	=	Hepatocyte nuclear factor 4
K1	=	Vitamin K1
K2/MK4	=	Vitamin K2/menaquinone
K3	=	Vitamin K3/menadione
KO	=	Knock out

MAPK = Mitogen-activated protein kinase

MGP = Matrix gla protein

MRP = Multidrug resistance-associated protein

PXE = Pseudoxanthoma elasticum

RPE = Retinal pigmented epithelium

VEGF = Vascular endothelial growth factor

VKOR = Vitamin K oxido-reductase

wt = Wild type

REFERENCES

[1] Rigal D. Observations pour servir á l'histoire de la cheloide diffuse xantholasmique. Ann Derm Syph 1881; 21: 491-501.

[2] Darier J. Pseudoxanthoma elasticum. Monatshefte Prakt Derm 1896; 23: 609-17.

[3] Grönblad E. Angioid streaks--pseudoxanthoma elasticum. Acta Ophthal 1929; 7: 329.

[4] Hu X, Plomp AS, van SS, Wijnholds J, de Jong PT, Bergen AA. Pseudoxanthoma elasticum: a clinical, histopathological, and molecular update. Surv Ophthalmol 2003; 48: 424-38.

[5] Walker ER, Frederickson RG, Mayes MD. The mineralization of elastic fibers and alterations of extracellular matrix in pseudoxanthoma elasticum. Ultrastructure, immunocytochemistry, and X-ray analysis. Arch Dermatol 1989; 125: 70-6.

[6] Baccarani-Contri M, Vincenzi D, Cicchetti F, Mori G, Pasquali-Ronchetti I. Immunochemical identification of abnormal constituents in the dermis of pseudoxanthoma elasticum patients. Eur J Histochem 1994; 38: 111-23.

[7] Limas C. Late onset focal dermal elastosis: a distinct clinicopathologic entity? Am J Dermatopathol 1999; 21: 381-3.

[8] Choi GS, Kang DS, Chung JJ, Lee MG. Osteoma cutis coexisting with cutis laxa-like pseudoxanthoma elasticum. J Am Acad Dermatol 2000; 43: 337-9.

[9] Nikko AP, Dunningan M, Cockerell CJ. Calciphylaxis with histologic changes of pseudoxanthoma elasticum. Am J Dermatopathol 1996; 18: 396-9.

[10] Gross G. Osteitis deformans (Paget)--angioid streaks (Knapp)--pseudoxanthoma elasticum (Darier)--manifestations of a common systemic disease?. Arch Orthop Unfallchir 1959; 50: 613-7.

[11] Vanakker OM, Martin L, Gheduzzi D, *et al*. Pseudoxanthoma elasticum-like phenotype with cutis laxa and multiple coagulation factor deficiency represents a separate genetic entity. J Invest Dermatol 2007; 127: 581-7.

[12] Finger RP, Charbel IP, Ladewig MS, *et al*. Pseudoxanthoma elasticum: genetics, clinical manifestations and therapeutic approaches. Surv Ophthalmol 2009; 54: 272-85.

[13] Dreyer R, Green WR. The pathology of angioid streaks: a study of twenty-one cases. Trans Pa Acad Ophthalmol Otolaryngol 1978; 31: 158-67.

[14] Lim JI, Bressler NM, Marsh MJ, Bressler SB. Laser treatment of choroidal neovascularization in patients with angioid streaks. Am J Ophthalmol 1993; 116: 414-23.

[15] Pece A, Avanza P, Galli L, Brancato R. Laser photocoagulation of choroidal neovascularization in angioid streaks. Retina 1997; 17: 12-6.

[16] Gragoudas ES, Adamis AP, Cunningham ET, Jr., Feinsod M, Guyer DR. Pegaptanib for neovascular age-related macular degeneration. N Engl J Med 2004; 351: 2805-16.

[17] Rosenfeld PJ, Brown DM, Heier JS, *et al*. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006; 355: 1419-31.

[18] Finger RP, Charbel IP, Ladewig M, Holz FG, Scholl HP. Intravitreal bevacizumab for choroidal neovascularisation associated with pseudoxanthoma elasticum. Br J Ophthalmol 2008; 92: 483-7.

[19] Navarro-Lopez F, Llorian A, Ferrer-Roca O, Betriu A, Sanz G. Restrictive cardiomyopathy in pseudoxanthoma elasticum. Chest 1980; 78: 113-5.

[20] Przybojewski JZ, Maritz F, Tiedt FA, van der Walt JJ. Pseudoxanthoma elasticum with cardiac involvement. A case report and review of the literature. S Afr Med J 1981; 59: 268-75.

[21] Challenor VF, Conway N, Monro JL. The surgical treatment of restrictive cardiomyopathy in pseudoxanthoma elasticum. Br Heart J 1988; 59: 266-9.

[22] Fukuda K, Uno K, Fujii T, Mukai M, Handa S. Mitral stenosis in pseudoxanthoma elasticum. Chest 1992; 101: 1706-7.

[23] Lebwohl M, Halperin J, Phelps RG. Brief report: occult pseudoxanthoma elasticum in patients with premature cardiovascular disease. N Engl J Med 1993; 329: 1237-9.

[24] Fah L. [Pseudoxanthoma elasticum--a visual diagnosis]. Schweiz Med Wochenschr 1991; 121: 660-3.

[25] Trip MD, Smulders YM, Wegman JJ, *et al*. Frequent mutation in the ABCC6 gene (R1141X) is associated with a strong increase in the prevalence of coronary artery disease. Circulation 2002; 106: 773-5.

[26] Koblos G, Andrikovics H, Prohaszka Z, Tordai A, Varadi A, Aranyi T. The R1141X loss-of-function mutation of the ABCC6 gene is a strong genetic risk factor for coronary artery disease. Genet Test Mol Biomarkers 2010; 14: 75-8.

[27] Le Saux O, Urban Z, Tschuch C, *et al*. Mutations in a gene encoding an ABC transporter cause pseudoxanthoma elasticum. Nat Genet 2000; 25: 223-7.

[28] Bergen AA, Plomp AS, Schuurman EJ, *et al*. Mutations in ABCC6 cause pseudoxanthoma elasticum. Nat Genet 2000; 25: 228-31.

[29] Ringpfeil F, Lebwohl MG, Christiano AM, Uitto J. Pseudoxanthoma elasticum: mutations in the MRP6 gene encoding a transmembrane ATP-binding cassette (ABC) transporter. Proc Natl Acad Sci USA 2000; 97: 6001-6.

[30] Pulkkinen L, Nakano A, Ringpfeil F, Uitto J. Identification of ABCC6 pseudogenes on human chromosome 16p: implications for mutation detection in pseudoxanthoma elasticum. Hum Genet 2001; 109: 356-65.

[31] Symmons O, Varadi A, Aranyi T. How segmental duplications shape our genome: recent evolution of ABCC6 and PKD1 Mendelian disease genes. Mol Biol Evol 2008; 25: 2601-13.

[32] Cai L, Struk B, Adams MD, *et al*. A 500-kb region on chromosome 16p13.1 contains the pseudoxanthoma elasticum locus: high-resolution mapping and genomic structure. J Mol Med 2000; 78: 36-46.

[33] Dauwerse JG, Wessels JW, Giles RH, *et al*. Cloning the breakpoint cluster region of the inv(16) in acute nonlymphocytic leukemia M4 Eo. Hum Mol Genet 1993; 2: 1527-34.

[34] van Dongen JJ, Macintyre EA, Gabert JA, *et al*. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia 1999; 13: 1901-28.

[35] Buys TP, Chari R, Lee EH, *et al*. Genetic changes in the evolution of multidrug resistance for cultured human ovarian cancer cells. Genes Chromosomes Cancer 2007; 46: 1069-79.

[36] Aranyi T, Ratajewski M, Bardoczy V, *et al*. Identification of a DNA methylation-dependent activator sequence in the pseudoxanthoma elasticum gene, ABCC6. J Biol Chem 2005; 280: 18643-50.

[37] Aranyi T, Faucheux BA, Khalfallah O, *et al*. The tissue-specific methylation of the human tyrosine hydroxylase gene reveals new regulatory elements in the first exon. J Neurochem 2005; 94: 129-39.

[38] Ratajewski M, Van de Ven WJ, Bartosz G, Pulaski L. The human pseudoxanthoma elasticum gene ABCC6 is transcriptionally regulated by PLAG family transcription factors. Hum Genet 2008; 124: 451-63.

[39] Ratajewski M, Bartosz G, Pulaski L. Expression of the human ABCC6 gene is induced by retinoids through the retinoid X receptor. Biochem Biophys Res Commun 2006; 350: 1082-7.

[40] Jiang Q, Matsuzaki Y, Li K, Uitto J. Transcriptional regulation and characterization of the promoter region of the human ABCC6 gene. J Invest Dermatol 2006; 126: 325-35.

[41] de Boussac H, Ratajewski M, Sachrajda I, *et al.* The ERK1/2-hepatocyte nuclear factor 4alpha axis regulates human ABCC6 gene expression in hepatocytes. J Biol Chem 2010; 285: 22800-8.

[42] Bolotin E, Liao H, Ta TC, *et al*. Integrated approach for the identification of human hepatocyte nuclear factor 4alpha target

genes using protein binding microarrays. Hepatology 2010; 51: 642-53.

[43] Odom DT, Zizlsperger N, Gordon DB, *et al*. Control of pancreas and liver gene expression by HNF transcription factors. Science 2004; 303: 1378-81.

[44] Sladek FM, Zhong WM, Lai E, Darnell JE, Jr. Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. Genes Dev 1990; 4: 2353-65.

[45] Kool M, van der LM, de HM, Baas F, Borst P. Expression of human MRP6, a homologue of the multidrug resistance protein gene MRP1, in tissues and cancer cells. Cancer Res 1999; 59: 175-82.

[46] Ratajewski M, de Boussac H, Sachrajda I, *et al*. A strong primate- and tissue-specific enhancer located in the first intron of ABCC6 gene is regulated by CCAAT/Enhancer binding protein. Biochemical J 2011; Submitted.

[47] Li Q, Jiang Q, Pfendner E, Varadi A, Uitto J. Pseudoxanthoma elasticum: clinical phenotypes, molecular genetics and putative pathomechanisms. Exp Dermatol 2009; 18: 1-11.

[48] Hamlin N, Beck K, Bacchelli B, Cianciulli P, Pasquali-Ronchetti I, Le Saux O. Acquired Pseudoxanthoma elasticum-like syndrome in beta-thalassaemia patients. Br J Haematol 2003; 122: 852-4.

[49] Edinger C, Bollt O, Le Saux O. Tissue-specific down regulation of *ABCC6* expression in beta-thalassemia mice. Ethnic Dis 2009; 19: 63-4.

[50] Schulz V, Hendig D, Henjakovic M, Szliska C, Kleesiek K, Gotting C. Mutational analysis of the ABCC6 gene and the proximal ABCC6 gene promoter in German patients with pseudoxanthoma elasticum (PXE). Hum Mutat 2006; 27: 831.

[51] Hendig D, Arndt M, Szliska C, Kleesiek K, Gotting C. SPP1 promoter polymorphisms: identification of the first modifier gene for pseudoxanthoma elasticum. Clin Chem 2007; 53: 829-36.

[52] Zarbock R, Hendig D, Szliska C, Kleesiek K, Gotting C. Pseudoxanthoma elasticum: genetic variations in antioxidant genes are risk factors for early disease onset. Clin Chem 2007; 53: 1734-40.

[53] Zarbock R, Hendig D, Szliska C, Kleesiek K, Gotting C. Vascular endothelial growth factor gene polymorphisms as prognostic markers for ocular manifestations in pseudoxanthoma elasticum. Hum Mol Genet 2009; 18: 3344-51.

[54] Schon S, Schulz V, Prante C, *et al*. Polymorphisms in the xylosyltransferase genes cause higher serum XT-I activity in patients with pseudoxanthoma elasticum (PXE) and are involved in a severe disease course. J Med Genet 2006; 43: 745-9.

[55] Ilias A, Urban Z, Seidl TL, *et al*. Loss of ATP-dependent transport activity in pseudoxanthoma elasticum-associated mutants of human ABCC6 (MRP6). J Biol Chem 2002; 277: 16860-7.

[56] Belinsky MG, Chen ZS, Shchaveleva I, Zeng H, Kruh GD. Characterization of the drug resistance and transport properties of multidrug resistance protein 6 (MRP6, ABCC6). Cancer Res 2002; 62: 6172-7.

[57] Madon J, Hagenbuch B, Landmann L, Meier PJ, Stieger B. Transport function and hepatocellular localization of mrp6 in rat liver. Mol Pharmacol 2000; 57: 634-41.

[58] Fulop K, Barna L, Symmons O, Zavodszky P, Varadi A. Clustering of disease-causing mutations on the domain-domain interfaces of ABCC6. Biochem Biophys Res Commun 2009; 379: 706-9.

[59] Dawson RJ, Hollenstein K, Locher KP. Uptake or extrusion: crystal structures of full ABC transporters suggest a common mechanism. Mol Microbiol 2007; 65: 250-7.

[60] Dawson RJ, Locher KP. Structure of a bacterial multidrug ABC transporter. Nature 2006; 443: 180-5.

[61] Aller SG, Yu J, Ward A, *et al*. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 2009; 323: 1718-22.

[62] Smith PC, Karpowich N, Millen L, *et al*. ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer. Mol Cell 2002; 10: 139-49.

[63] Klement JF, Matsuzaki Y, Jiang QJ, *et al*. Targeted ablation of the abcc6 gene results in ectopic mineralization of connective tissues. Mol Cell Biol 2005; 25: 8299-310.

[64] Gorgels TG, Hu X, Scheffer GL, *et al*. Disruption of Abcc6 in the mouse: novel insight in the pathogenesis of pseudoxanthoma elasticum. Hum Mol Genet 2005; 14: 1763-73.

[65] Meng H, Vera I, Che N, *et al*. Identification of Abcc6 as the major causal gene for dystrophic cardiac calcification in mice through integrative genomics. Proc Natl Acad Sci USA 2007; 104: 4530-5.

[66] Aherrahrou Z, Doehring LC, Ehlers EM, Liptau H, Depping R, Linsel-Nitschke P, *et al*. An alternative splice variant in Abcc6, the gene causing dystrophic calcification, leads to protein deficiency in C3H/He mice. J Biol Chem 2008; 283: 7608-15.

[67] Li Q, Sadowski S, Frank M, *et al*. The Abcc6a gene expression is required for normal zebrafish development. J Invest Dermatol 2010; 130: 2561-8.

[68] Uitto J, Pulkkinen L, Ringpfeil F. Molecular genetics of pseudoxanthoma elasticum: a metabolic disorder at the environment-genome interface? Trends Mol Med 2001; 7: 13-7.

[69] Jiang Q, Endo M, Dibra F, Wang K, Uitto J. Pseudoxanthoma elasticum is a metabolic disease. J Invest Dermatol 2009; 129: 348-54.

[70] Jiang Q, Oldenburg R, Otsuru S, Grand-Pierre AE, Horwitz EM, Uitto J. Parabiotic heterogenetic pairing of abcc6-/-/rag1-/- mice and their wild-type counterparts halts ectopic mineralization in a murine model of pseudoxanthoma elasticum. Am J Pathol 2010; 176: 1855-62.

[71] Macmillan DC, Vickers HR. Pseudoxanthoma elasticum and a coagulation defect. Br J Dermatol 1971; 84: 182.

[72] Rongioletti F, Bertamino R, Rebora A. Generalized pseudoxanthoma elasticum with deficiency of vitamin K-dependent clotting factors. J Am Acad Dermatol 1989; 21: 1150-2.

[73] Le Corvaisier-Pieto C, Joly P, Thomine E, Lair G, Lauret P. [Generalized pseudoxanthoma elasticum combined with vitamin K dependent clotting factors deficiency]. Ann Dermatol Venereol 1996; 123: 555-8.

[74] Berkner KL. Vitamin K-dependent carboxylation. Vitam Horm 2008; 78: 131-56.

[75] Oldenburg J, Marinova M, Muller-Reible C, Watzka M. The vitamin K cycle. Vitam Horm 2008; 78: 35-62.

[76] Schurgers LJ, Teunissen KJ, Knapen MH, *et al*. Novel conformation-specific antibodies against matrix gamma-carboxyglutamic acid (Gla) protein: undercarboxylated matrix Gla protein as marker for vascular calcification. Arterioscler Thromb Vasc Biol 2005; 25: 1629-33.

[77] Schurgers LJ, Spronk HM, Skepper JN, *et al*. Post-translational modifications regulate matrix Gla protein function: importance for inhibition of vascular smooth muscle cell calcification. J Thromb Haemost 2007; 5: 2503-11.

[78] Schurgers LJ, Cranenburg EC, Vermeer C. Matrix Gla-protein: the calcification inhibitor in need of vitamin K. Thromb Haemost 2008; 100: 593-603.

[79] Jiang Q, Li Q, Uitto J. Aberrant mineralization of connective tissues in a mouse model of pseudoxanthoma elasticum: systemic and local regulatory factors. J Invest Dermatol 2007; 127: 1392-402.

[80] Li Q, Jiang Q, Schurgers LJ, Uitto J. Pseudoxanthoma elasticum: reduced gamma-glutamyl carboxylation of matrix gla protein in a mouse model (Abcc6-/-). Biochem Biophys Res Commun 2007; 364: 208-13.

[81] Gheduzzi D, Boraldi F, Annovi G, *et al*. Matrix Gla protein is involved in elastic fiber calcification in the dermis of pseudoxanthoma elasticum patients. Lab Invest 2007; 87: 998-1008.

[82] Vanakker OM, Martin L, Schurgers LJ, *et al*. Low serum vitamin K in PXE results in defective carboxylation of mineralization inhibitors similar to the GGCX mutations in the PXE-like syndrome. Lab Invest 2010; 90: 895-905.

[83] Borst P, van de WK, Schlingemann R. Does the absence of ABCC6 (multidrug resistance protein 6) in patients with Pseudoxanthoma elasticum prevent the liver from providing sufficient vitamin K to the periphery? Cell Cycle 2008; 7: 1575-9.

[84] Li J, Lin JC, Wang H, *et al*. Novel role of vitamin k in preventing oxidative injury to developing oligodendrocytes and neurons. J Neurosci 2003; 23: 5816-26.

[85] Garcia-Fernandez MI, Gheduzzi D, Boraldi F, *et al*. Parameters of oxidative stress are present in the circulation of PXE patients. Biochim Biophys Acta 2008; 1782: 474-81.

[86] Li Q, Jiang Q, Uitto J. Pseudoxanthoma elasticum: oxidative stress and antioxidant diet in a mouse model (Abcc6-/-). J Invest Dermatol 2008; 128: 1160-4.

[87] Thijssen HH, Vervoort LM, Schurgers LJ, Shearer MJ. Menadione is a metabolite of oral vitamin K. Br J Nutr 2006; 95: 260-6.

[88] Okano T, Shimomura Y, Yamane M, *et al*. Conversion of phylloquinone (Vitamin K1) into menaquinone-4 (Vitamin K2) in mice: two possible routes for menaquinone-4 accumulation in cerebra of mice. J Biol Chem 2008; 283: 11270-9.

[89] Harrington DJ, Soper R, Edwards C, Savidge GF, Hodges SJ, Shearer MJ. Determination of the urinary aglycone metabolites of vitamin K by HPLC with redox-mode electrochemical detection. J Lipid Res 2005; 46: 1053-60.

[90] Jiang Q, Li Q, Grand-Pierre AE, Schurgers LJ, Uitto J. Administration of Vitamin K Does Not Counteract the Ectopic Mineralization of Connective Tissues in *Abcc6-/-* Mice, a Model for Pseudoxanthoma Elasticum. Cell Cycle 2011; 10: 701-7.

[91] Brampton C, Yamaguchi Y, Vanakker OM, *et al*. An increase in dietary vitamin K does not prevent soft tissue mineralization in a mouse model of PXE. Cell Cycle 2011; submitted.

[92] Hendig D, Langmann T, Kocken S, *et al*. Gene expression profiling of ABC transporters in dermal fibroblasts of pseudoxanthoma elasticum patients identifies new candidates involved in PXE pathogenesis. Lab Invest 2008; 88: 1303-15.

[93] Beck K, Hayashi K, Dang K, Hayashi M, Boyd CD. Analysis of ABCC6 (MRP6) in normal human tissues. Histochem Cell Biol 2005; 123: 517-28.

[94] Boraldi F, Quaglino D, Croce MA, *et al*. Multidrug resistance protein-6 (MRP6) in human dermal fibroblasts. Comparison between cells from normal subjects and from Pseudoxanthoma elasticum patients. Matrix Biol 2003; 22: 491-500.

[95] Vanakker OM, Leroy BP, Coucke P, *et al*. Novel clinico-molecular insights in pseudoxanthoma elasticum provide an efficient molecular screening method and a comprehensive diagnostic flowchart. Hum Mutat 2008; 29: 205.

[96] Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002; 298: 1911-2.

[97] Sugden PH, Clerk A. Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors. Cell Signal 1997; 9: 337-51.

[98] Junttila MR, Li SP, Westermarck J. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. FASEB J 2008; 22: 954-65.

[99] Roberts PJ, Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene 2007; 26: 3291-310.

[100] Friday BB, Adjei AA. Advances in targeting the Ras/Raf/MEK/Erk mitogen-activated protein kinase cascade with MEK inhibitors for cancer therapy. Clin Cancer Res 2008; 14: 342-6.

[101] Pasquali-Ronchetti I, Garcia-Fernandez MI, Boraldi F, *et al*. Oxidative stress in fibroblasts from patients with pseudoxanthoma elasticum: possible role in the pathogenesis of clinical manifestations. J Pathol 2006; 208: 54-61.

[102] Reddy S, Yang W, Taylor DG, *et al*. Mitogen-activated protein kinase regulates transcription of the ApoCIII gene. Involvement of the orphan nuclear receptor HNF4. J Biol Chem 1999; 274: 33050-6.

[103] Hatzis P, Kyrmizi I, Talianidis I. Mitogen-activated protein kinase-mediated disruption of enhancer-promoter communication inhibits hepatocyte nuclear factor 4alpha expression. Mol Cell Biol 2006; 26: 7017-29.

[104] Xie X, Liao H, Dang H, *et al*. Down-regulation of hepatic HNF4alpha gene expression during hyperinsulinemia via SREBPs. Mol Endocrinol 2009; 23: 434-43.

[105] Martinez-Jimenez CP, Kyrmizi I, Cardot P, Gonzalez FJ, Talianidis I. HNF4{alpha} coordinates a transcription factor network regulating hepatic fatty acid metabolism. Mol Cell Biol 2010; 30: 565-77.

[106] Bailly A, Torres-Padilla ME, Tinel AP, Weiss MC. An enhancer element 6 kb upstream of the mouse HNF4alpha1 promoter is activated by glucocorticoids and liver-enriched transcription factors. Nucleic Acids Res 2001; 29: 3495-505.

[107] Hayashi H, Sugiyama Y. 4-phenylbutyrate enhances the cell surface expression and the transport capacity of wild-type and mutated bile salt export pumps. Hepatology 2007; 45: 1506-16.

[108] Loo TW, Clarke DM. Correction of defective protein kinesis of human P-glycoprotein mutants by substrates and modulators. J Biol Chem 1997; 272: 709-12.

[109] Egan ME, Pearson M, Weiner SA, *et al*. Curcumin, a major constituent of turmeric, corrects cystic fibrosis defects. Science 2004; 304: 600-2.

[110] Rubenstein RC, Egan ME, Zeitlin PL. *In vitro* pharmacologic restoration of CFTR-mediated chloride transport with sodium 4-phenylbutyrate in cystic fibrosis epithelial cells containing delta F508-CFTR. J Clin Invest 1997; 100: 2457-65.

[111] Rubenstein RC, Zeitlin PL. Sodium 4-phenylbutyrate downregulates Hsc70: implications for intracellular trafficking of DeltaF508-CFTR. Am J Physiol Cell Physiol 2000; 278: C259-67.

[112] Rubenstein RC, Zeitlin PL. A pilot clinical trial of oral sodium 4-phenylbutyrate (Buphenyl) in deltaF508-homozygous cystic fibrosis patients: partial restoration of nasal epithelial CFTR function. Am J Respir Crit Care Med 1998; 157: 484-90.

[113] Batshaw ML, MacArthur RB, Tuchman M. Alternative pathway therapy for urea cycle disorders: twenty years later. J Pediatr 2001; 138: S46-S54.

[114] Yam GH, Gaplovska-Kysela K, Zuber C, Roth J. Sodium 4-phenylbutyrate acts as a chemical chaperone on misfolded myocilin to rescue cells from endoplasmic reticulum stress and apoptosis. Invest Ophthalmol Vis Sci 2007; 48: 1683-90.

[115] Le Saux O, Fülöp K, Yamaguchi Y, *et al*. Assisted maturation of human *ABCC6* disease causing mutants in mouse liver. Hepatology 2011; submitted.

[116] Barton-Davis ER, Cordier L, Shoturma DI, Leland SE, Sweeney HL. Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of mdx mice. J Clin Invest 1999; 104: 375-81.

[117] Xi B, Guan F, Lawrence DS. Enhanced production of functional proteins from defective genes. J Am Chem Soc 2004; 126: 5660-1.

[118] Welch EM, Barton ER, Zhuo J, *et al*. PTC124 targets genetic disorders caused by nonsense mutations. Nature 2007; 447: 87-91.

Received: January 23, 2010 Revised: March 27, 2010 Accepted: March 27, 2010

Targeting CFTR: How to Treat Cystic Fibrosis by CFTR-Repairing Therapies

Margarida D. Amaral[*,†]

University of Lisboa, Faculty of Sciences, BioFIG, Centre for Biodiversity, Functional and Integrative Genomics, Portugal

Centre of Human Genetics, National Institute of Health, Lisboa, Portugal

Abstract: Several novel compounds recently appeared as promising leads to develop effective drugs against the basic defect in Cystic fibrosis (CF) and the first rationale therapies for CF relying on the understanding of the basic defect started to hit the clinical setting.

Most of these efforts are focused on correcting the F508del mutation (occurring in ~90% of CF patients) which causes misfolding of the CF transmembrane conductance regulator (CFTR) protein, the intracellular retention of such abnormal conformation by the endoplasmic reticulum quality control and premature degradation, thus precluding CFTR from reaching the cell membrane where it normally functions as a cAMP-stimulated Cl^- channel.

Here, several rationale therapeutic strategies are briefly reviewed, namely, mutation-specific (or "CFTR-repairing") approaches (with a particular focus on the cellular defect associated with F508del-CFTR), manipulation of other ionic (non-CFTR) conductances and gene therapy. Still more innovative strategies, such as manipulation of the proteostasis network, displacement of molecular chaperones, targeting mutant CFTR by *in silico* small-molecule screens and systems biology approaches are also discussed.

Keywords: Basic defect, CFTR modulator, corrector, F508del, drug discovery.

INTRODUCTION

Cystic Fibrosis and CFTR

Cystic Fibrosis (CF) is the most common, lethal monogenic disorder, estimated to affect 30,000 individuals in the European Union and about the same number in the US [1], with an incidence of 1 per 2,500-4,000 newborns. Clinically, CF is dominated by the involvement of the respiratory tract, the main cause of morbidity and mortality, through airway obstruction by thick mucus and chronic infections, especially by *Pseudomonas aeruginosa*, eventually leading to loss of the respiratory function [2]. Other CF symptoms include pancreatic dysfunction, elevated sweat electrolytes, male infertility, intestinal obstruction, liver cirrhosis, etc. [3]. However, there is wide clinical variability in organ involvement from classical severe forms to milder and atypical presentations [4]. Despite impressive advances in relating the molecular basis of CF to organ-level disease, CF is still a very limiting disorder, both in terms of life expectancy (37 yrs in the US) and quality of life [5].

The disease is caused by dysfunction of a single gene, spanning a genomic region of ~190 Kb and consisting of 27 exons, which codes for the CF transmembrane conductance regulator (CFTR) protein [6], a cAMP-activated chloride (Cl-) channel, expressed at the apical membrane of most surface epithelial cells lining the airways, the gastrointestinal tract and the ducts of several glands, like the pancreas, airways submucosal and sweat glands [7]. Due to the absence/ impairment of functional CFTR, very little or no CFTR-mediated Cl^- transport is detected in CF epithelia. Various other ion transporters are also regulated by CFTR, above all the epithelial Na^+ channel (ENaC) - a major regulator of salt and water re-absorption which is downregulated by active CFTR [8, 9]. In turn, this ENaC hyperabsorption which results from defective/ lack of CFTR, has been postulated to lead to water depletion from the airway surface liquid (ASL) in CF and consequent reduction in its height and fluidity [10]. This phenomenon is believed to contribute to the deleterious cascade of mucus accumulation, infection, inflammation and destruction that characterizes CF lung disease [11].

CFTR itself (also ABCC7), a member of the ABC transporter superfamily, is a large multidomain protein comprising 1480 amino acid residues arranged in two membrane spanning domains (MSD1/2), two nucleotide (ATP) binding domains (NBD1/2) and a unique highly polar regulatory domain (RD) with multiple putative phosphorylation sites [12]. For correct appearance of functional CFTR at the surface of epithelial cells, multiple cellular processes have to occur in an accurate and coordinated way. These start by the production and splicing of the long CFTR primary transcript, followed by correct processing of the protein along the secretory pathway and insertion in the apical membrane and ending in the adequate activation of the channel in a tightly regulated fashion.

Each of the numerous CFTR gene mutations so far reported to cause CF [13] disrupt protein function in distinct ways and by affecting one or more of these processes. In order to tackle all these different gene mutations towards

*Address correspondence to this author at the Faculty of Sciences, University of Lisboa, Campo Grande-C8, 1749-016 Lisboa, Portugal; Tel: +351-21-750 81 55; Fax: +351-21-750 00 88; E-mail: mdamaral@fc.ul.pt

[†]**Current address:** EMBL Heidelberg, Meyerhofstraße 1, 69117 Heidelberg, Germany

1389-4501/11 $58.00+.00 **© 2011 Bentham Science Publishers Ltd.**

corrective (or "CFTR-repairing") therapy, they have been grouped into functional classes. However, before describing this classification of mutations and respective therapeutic strategies (see below under "*Functional Classes of CFTR Mutations and "CFTR-Repairing Therapies*"), a brief overview of the mutations themselves as well as their molecular and cellular consequences is provided hereunder.

CFTR Gene Mutations

More than 1,500 alterations have been described to date in the CFTR gene [13], the majority of which are pathologic. They have the following distribution: missense (41.5%); frameshift (16%); splicing (12.5%); nonsense (9.5%); large (3%) and in-frame (2%) deletions/ insertions; and promoter (0.5%); plus 15% of presumed non-pathological variants [13]. However, the most common cause of CF is a deletion of 3 nucleotides resulting in loss of phenylalanine at position 508 of the polypeptidic chain [14, 15]. This mutation, termed F508del and accounting for ~70% of CF chromosomes worldwide, occurs in ~90% of CF patients in at least one allele and is associated with a severe clinical phenotype [16]. Generally, a higher frequency of this mutation is observed in Northern *vs*. Southern European populations [17, 18].

Such a wide spectrum of mutations in the CFTR gene comprises a large number of uncommon variants for which disease prognosis is difficult, also because the underlying functional defect is unclear [19]. Many of these uncommon variants still result in partially functional CFTR and hence milder or "atypical" forms of CF [20]. However, clinical phenotypes may vary widely for the same genotype, thus posing considerable challenges in establishing diagnosis and prognosis of CF in relation to genotypes [5, 19, 20]. In fact, it has been estimated that overall, 7% of CF patients are not diagnosed until age 10 years, with a significant proportion not diagnosed until after age 15 years [20]. The phenotypical unpredictability of these "milder" mutations poses even greater challenges to the establishment of newborn screening programmes which are based on DNA analysis, especially in countries (e.g. Southern Europe) where their proportion is higher. These challenges include above all the choice of mutations to be included in the "screening panel", due to major difficulties in establishing a clear follow-up procedure in positive cases for these unpredictable mutations.

Functional Classes of CFTR Mutations and "CFTR-Repairing" Therapies

Since ultimately, all disease-causing CFTR mutations result in defective cAMP-regulated Cl^- secretion by epithelial cells [21, 22], in principle, fixing the basic defect to restore CFTR-mediated Cl^- transport should clinically improve CF. Moreover, once the underlying defect(s) of CFTR mutants are known, they can be grouped into functional classes so as to be tackled by similar corrective strategies, and hopefully with the same compounds (see below). The previously defined functional classes of CFTR mutations, which have been explained in detail elsewhere [21, 22], are also briefly described below.

Class I mutations impair protein production, often being nonsense mutations (with premature stop codons, e.g. G542X or W1282X) usually leading to mRNA degradation by nonsense-mediated decay (NMD). Class II mutations (where F508del is included) affect CFTR protein processing due to endoplasmic reticulum (ER) retention by the ER quality control (ERQC) and degradation [23]. Class III mutants (e.g., G551D) traffic to the cell membrane but exhibit impaired gating by disrupting channel regulation. Class IV mutations (e.g., R334W) decrease channel conductance, i.e., flow of Cl^- ions, usually by locating in the channel conducting pore. Class V mutations significantly reduce normal protein levels, often by affecting splicing (3272-26A>G or 3849 + 10 kb C>T) and generating both aberrant and normal transcripts, the levels of which vary among different CF patients with the same mutation and correlate with disease severity [24, 25].

The next step, after examining the molecular and cellular basis of CF mutations, is to design appropriate and effective treatments to correct these basic molecular and cellular defects. Some of these "CFTR-repairing" (or "CFTR-assist") therapeutic strategies [1, 11], are currently under experimental testing or already progressed to the clinical setting. Since they have been reviewed elsewhere recently [11, 26], only a brief overview of these approaches is provided here.

- Class I. Aminoglycoside antibiotics and synthetic novel small molecules have been described to camouflage premature termination codons (PTCs) and suppress translational fidelity by allowing the incorporation of an amino acid. This permits translation to continue over PTCs to the normal termination of the transcript thus producing full-length protein. Examples of clinical and pre-clinical trials carried out in patients with CF to test correction of CFTR function by translational "read-through" include both gentamicin [27] and more recently ribosome-binding molecules like Ataluren (formerly PTC124, PTC Therapeutics) [28, 29]. More recently, novel geneticin analogs have been developed and shown *in vitro* to be of promising value [30].
- Class II. Chemical (unspecific) and pharmacological (specific) chaperones are small molecules which can potentially bind more or less specifically to mutant CFTR proteins, promoting their folding and stability (for a review see [23]). Both these types of compounds allow mutants to escape ER degradation and reach the cell surface (see below "*Targeting the Cellular Defect Associated with F508del-CFTR*"). Among these compounds, termed in the CF field as "correctors" [31], there are currently promising candidate drugs in trial to correct the F508del defect in patients. Such is the case of VX-809 (Vertex Pharmaceuticals) for which a Phase IIa clinical trial was recently completed. Although the study was primarily designed to test safety and tolerability of the drug some reduction in sweat chloride values were reported [32].
- Class III. CFTR activators such as the flavonoid genistein [33] which can overcome the channel gating/ regulation defects of CFTR mutants which already localize to the cell membrane [34], are termed in the CF field as "potentiators" [35]. Although a CFTR potentiator is a compound which increases Cl^- secretion only in the background of the normal

physiological control, i.e., after stimulation of the cAMP/PKA signalling pathway, an ideal potentiator should not modulate this pathway, but rather act on the channel itself. Among potentiators, one of the most promising drug candidates which has fulfilled such criterion, is VX-770 (Vertex Pharmaceuticals) [36]. This compound revealed a 10% improvement in lung function after 2 weeks of twice-daily therapy in a Phase IIa trial involving patients bearing the G551D in at least one CFTR allele [37]. Two Phase III studies (one for paediatric and another for adolescent/ adult patients) began in Summer 2009. Currently, a VX-770 clinical trial is also recruiting CF patients aged 12 yrs and older who are F508del-homozygous, since past studies have revealed that a few of these patients have some F508del-CFTR protein at the membrane of native respiratory cells [38, 39], albeit expected to display much reduced function [40, 41].

- Class IV. Compensation for reduced CFTR conductance can be achieved either by increasing the overall cell surface density of these mutants with class II correctors or/and through increased stimulation of the existing channels with class III potentiators.
- Class V. Increasing the levels of splicing factors that correct missplicing or manipulating those that alter the balance of different splice forms, are valid strategies to promote increased levels of correctly spliced transcripts in CF patients bearing CFTR splicing mutations (~10% of all CF patients). Knowledge of the factors required to manipulate mRNA splicing in the desired direction, combined with recent advances in the delivery of "splice switching" oligomers to cells, holds great promise for the development of new therapeutic strategies for the treatment of splicing mutations occurring in various diseases [42]. However, despite the demonstrated success of such strategies in cell culture this type of approach has not yet moved into clinical trials. It is nevertheless, expected that CFTR potentiators with proven efficacy on wt-CFTR will also move soon into the clinical setting for patients with class V mutations.

Despite the attractiveness of this rationale for "class-approach" for CFTR-repairing therapy, it should be emphasized that, for many of the very high number of CF-causing mutations so far reported in the CFTR gene, the corresponding functional defect remains unknown, thus precluding their functional classification. The CFTR2 project [43], a recent major research effort aimed at understanding the defects associated with a significant proportion of CFTR mutants at the molecular, cellular and functional levels for disease diagnosis and prognosis, will allow such classification of CFTR mutations, thus enabling considerable progress towards mutation-specific therapies. Indeed, by creating isogenic epithelial cell lines each expressing a different CFTR variant, CFTR2 will constitute a valuable resource for the development of targeted compounds by "CFTR-repairing" strategies (see below) [11]. Moreover, this initiative will also provide key insights into the structure and function of CFTR.

Although appealing, application of mutation-specific therapeutic strategies to CF patients with different CFTR genotypes still has its caveats. In fact, although sharing the same molecular/cellular defect(s), CFTR mutations in the same functional class may respond differently to a given compound. For instance, bisaminomethylbithiazoles, which were shown to correct F508del-CFTR in F508del/F508del human bronchial epithelia, did not correct another temperature-sensitive trafficking mutant, P574H-CFTR [31]. Another example is gentamicin which appears to correct W1282X more efficiently [44] than other nonsense CFTR mutations [45]. Similar discrepancies in efficacy are expected to other compounds when tested on different CFTR mutants. In this respect, the CFTR2 resources (see above) will be extremely helpful, since such collection of cell lines expressing different CFTR variants can also be used to test pre-clinically the efficacy of "CFTR-repairing" compounds on different genotypes before moving into clinical trials.

Manipulating other Ionic Conductances to Restore Airway Surface Liquid

Since CF airways also exhibit a major enhancement of sodium (Na^+) absorption and subsequent ASL dehydration, another therapeutic approach to CF, which is completely different from the above because it is independent of the CFTR genotype, relies on the correction of ion transport which is not mediated by CFTR. This is the so-called "bypassing approach" (reviewed in [11, 46]).

This strategy proposes for instance to correct for the Na^+ hyperabsorption by inhibiting the Na^+ epithelial channel (ENaC) and/or for the lack of CFTR-mediated Cl^- transport, by activating other non-CFTR Cl^- channels, such as the calcium (Ca^{2+})-activated Cl^- channels (CaCCs) [11].

To correct for the Na^+ hyperabsorption in the airways of CF patients, early clinical trials were conducted with aerosolized form the specific ENaC blocker amiloride, and demonstrating that this compound improved mucociliary clearance (MC) and expanded airway surface liquid (ASL) CF cells [47, 48]. Despite these encouraging results, the low potency and rapid absorption of amiloride by airway epithelia translated into a short duration of efficacy for CF patients. Subsequent trials have thus tested improved ENaC blockers, also as aerosolized therapies for CF, alone or in combination with hypertonic saline, and encouraging results have been reported [49] (for a review on ENaC therapy see [50]). Since ENaC activation has been shown to depend on several trypsin-family serine peptidases [51, 52], therapeutic approaches based on protease inhibitors are also being pursued [53]. Another approach to selectively downregulate ENaC involves usage of small interference (si) RNAs to downeregulate its expression levels [54-56] and, given the rapid developments in siRNA therapeutics targeted to the lung [57], this strategy may become feasible in the short-term. However, since ENaC cannot be fully blocked, given the associated risk of hypotension and other symptoms associated with psuedohypoaldosteronism (PHA1), all ENaC targeted therapies must might under tight control.

As to CaCCs, after more than 25 years searching for possible candidates, the molecular identity of the latter was finally recognized as being one member of the anoctamin family of transmembrane proteins (ANO1) or transmembrane protein 16A (TMEM16A) [58-60].

The current most promising therapeutic strategy for alternative Cl^- conductances is to stimulate CaCCs through ATP-activated purinergic receptors P2Y(2) using synthetic nucleotides which are more stable and selective than ATP, such as INS365 or denufosol tetrasodium (INS37217, Inspire Pharmaceuticals). The latter, a metabolically more stable and potent compound, has the advantage of not only stimulating CaCC channels but also of inhibiting ENaC, albeit modestly, thus attenuating excessive Na^+ absorption and ASL dehydration [11, 46]. Denufosol already finished a first Phase III (TIGER-1) clinical trial [61], demonstrating statistical significance for lung function (FEV1) improvement. Currently, CF patients are being enrolled for a second Phase 3 trial (TIGER-2) with this drug candidate. These trials started before identification of TMEM16A, so it remains to be elucidated that this is actually the target of denufosol.

Similarly to the bypassing approach that ignores the basic CFTR defect, other recent therapeutic strategies have focused on correcting other non-CFTR defects. One example is the age-dependent accumulation of ceramide, described to be due to enhanced pH-dependent sphingomyelinase activity, which cleaves sphingomyelin to ceramide [62]. Extreme function of this enzyme and excessive accumulation of ceramide were shown to occur in the respiratory tract of uninfected *cftr-/-* mice, resulting in constitutive pulmonary inflammation and death of respiratory epithelial cells [62]. Although still controversial that such defects are primary in the CF pathogenesis cascade [63, 64], pharmacological treatment of these mice with the sphingomyelinase blocker amitriptyline (an antidepressant drug in previous clinical use) normalizes pulmonary ceramide levels and prevents other pathological findings, including susceptibility to infection [62].

Gene Therapy

To date, gene therapy has failed to demonstrate a clinical benefit for CF after repeated administration. Notwithstanding, a lot was gained from the pre-clinical and clinical studies that were performed, not only in endpoints for efficacy assessment [65, 66] but also in key knowledge of the basic biology of the epithelium that is essential for a better understanding the CF pathophysiology.

One major problem of single-dose gene therapy achieved by vectors that insert its CFTR transgene into the hosting cell (e.g., lentiviruses) is that cells lose expression of the "intruding" gene after some time, lasting only for 4-6 weeks. This is mostly due to usage of a "shorter version" of the CFTR gene (called complementary DNA, or cDNA) which is only ~6kb instead of the long (~190 kb), full genomic CFTR gene. The absence of intercalating regions (introns) in such "shorter gene versions" leads to silencing of the genes by complex mechanisms, still not fully elucidated. Thus, one plausible alternative is to use larger CFTR constructions which, however, due to their large size cannot be inserted into conventional vectors. Human artificial chromosomes (HACs) containing half of the CFTR genomic sequence were already inserted into mammalian cells by suicidal bacteria and shown to maintain CFTR expression over 50 cell generations [67]. More recently, successful generation of genomic constructs containing the full-length CFTR gene were also reported [68]. Although with still many barriers to overcome, such an approach may still hold promise of gene therapy success for CF.

Targeting the Cellular Defect Associated with F508del-CFTR

Since the F508del mutation is the main cause of CF, being the most prevalent in patients worldwide, major research efforts aim to understand and correct this mutant. F508del is a class II mutant (see above), so the major defect is causes on CFTR protein is its intracellular localization [15, 69]. Indeed, F508del-CFTR fails to traffic to the plasma membrane due to major retention at the level of the ER and degradation by a process known as ERAD (ER-associated degradation). This ER retention is believed to result from its failure in acquiring a native (folded) conformation. Structural cues exposed in the mutant protein are somehow recognized by the ER quality control (ERQC), the "sensor" of ERAD a mechanism that reduces levels of misfolded proteins in the secretory pathway through proteasomal degradation via the ubiquitin-(Ub)proteasomal pathway (UPP) (reviewed in [70]). Since it would be of potential therapeutic benefit to render the ERQC less stringent towards F508del-CFTR, many groups, including ourselves have focused their efforts on the identification of the key specific intervenients responsible for recognizing and sending most of this mutant to degradation. Since this approach has been excellently reviewed recently [12], it is only briefly mentioned here. Some more novel strategies are described in the following section.

According to our own working model of the ERQC [71, 72] several sequential checkpoints involving distinct protein machineries (mostly molecular chaperones) assess the folding status of newly synthesized proteins targeting for degradation those which cannot achieve a folded conformation in a timely fashion. Targeting the components of these machineries for therapeutic benefit (reviewed in [23]) has been attempted soon after the first molecular chaperone, Hsp70, was identified to associate with CFTR [73]. The compound then used to attempt F508del-CFTR rescuing was the polyamine deoxyspergualin [74], described to bind both molecular chaperones Hsp70 and Hsp90 and also a potent immunosuppressive, clinically applied for graft rejection protection by a yet unknown mechanism [75]. However, the effect, which was then described for this compound in rescuing the processing of F508del-CFTR, could not be confirmed in another study [76]. Moreover, it was subsequently shown that disruption of the CFTR/ Hsp90 association with geldanamycin, an Hsp90-specific benzoquinone ansamycin drug, prevented maturation of wt-CFTR and accelerated its degradation by the proteasome [77]. Dissociation from Hsp90 thus seems detrimental to the efficiency of CFTR processing.

Similarly to Hsp90, decreased wt-CFTR processing was also found by downregulating the levels of the ER membrane-bound and Ca^{2+}-dependent chaperone/lectin calnexin, the major component of another proposed ERQC checkpoint, or by preventing its interaction with CFTR, by devoiding CFTR of the glycan residues responsible such interaction [71]. Curiously, and despite the recent confirmation of these data by additional studies [78-80], some groups reported to have achieved "release" of F508del-CFTR from the ER by

perturbing interactions with calnexin, or proposed this to be the MoA for the effects brought about by diverse compounds claimed to cause some F508del-CFTR maturation. Such compounds include the Ca^{2+}-pump inhibitors (depleting Ca^{2+} from the ER) thapsigargin [81] and curcumin, the major component of tumergic [82], and N-butyldeoxynojirimycin (miglustat) an inhibitor of ER α-glucosidases I and II [83, 84]. However, most of these results are controversial because rescue of F508del-CFTR by these compounds could not be confirmed by other groups as discussed previously [11, 12]. Importantly, miglustat (Zavesca®, Actelion) used in Gaucher disease therapy as an inhibitor of ceramide-specific glycosyltransferases [85] was assessed in a Phase IIa clinical trial involving F508del-CF patients. Although the trial was completed in Summer 2009, the results have not yet been released.

While the mechanism of action (MoA) of CFTR correctors is largely unknown, a recent study indicates that, at least some of them, may indeed compete with molecular chaperones such as Hsp70. In this study [86] Hsp70 was shown to bind to F508del-NBD1 of CFTR with 5-fold higher affinity than to the wild-type (wt) domain and that such high affinity was significantly decreased by pre-incubation with the CFTR corrector 4a [31]. Such decrease in affinity plausibly resulted from the ability of corrector 4a to mask some hydrophobic pocket(s) exposed in the surface of F508del-NBD1, thus increasing its (apparent) intrinsic folding. Speculation about the implications of these *in vitro* results to the *in vivo* scenario of full-length CFTR, seems to lead to the conclusion that corrector 4a does not merely "release" misfolded F508del-CFTR from Hsp70, leaving it "vulnerable" to other clearance mechanisms, but instead it promotes F508del-CFTR folding, even if apparently and as measured by the decreased exposure of hydrophobic surfaces.

Accordingly, to achieve rescuing of F508del-CFTR, it does not seem to be enough for small molecules to disrupt its association with molecular chaperones. It appears also crucial that the "released" protein has acquired a folded conformation (or some form of "protection" for its hydrophobic surfaces) to avoid instability and degradation. This is indeed the role of Hsp70 and chaperones in general, which are known to protect their "client proteins" from multiple interactions in the cell *milieu*, so that they can achieve their native conformation. However, since chaperones are know to exert dual effects on their substrates [87], they also to drastically target them for degradation if they are unable to accomplish so in a timely fashion.

Despite many more of less unsuccessful attempts to overcome the intracellular retention of F508del-CFTR, such manoeuvre would be of great therapeutic value, since this mutant is still functional. Nevertheless, it should be born in mind that when rescued to the cell surface by low temperature, for instance, its activity was found to be only ~20% of normal CFTR [40, 41]. Moreover, after low-temperature rescuing F508del-CFTR evidences a major instability at the cell membrane [88, 89] resulting from both enhanced endocytosis [90] and decreased recycling [91]. These instability and "vulnerability" to degradation of F508del-CFTR in distal secretory compartments also suggests that low temperature probably does not substantially increase its folding for the above-discussed reasons.

Consequently, to correct for the various defects associated with F508del-CFTR, two kinds of "correctors" are being searched for, namely by high-throughput (HT) approaches: 1) those rescuing the trafficking defect, but still relying on additional compounds to stabilize the protein at the cell surface and/or to increase channel activity; and 2) those overcoming the trafficking defect, but also achieving cell surface stabilization and enhanced Cl^- transport. The latter are probably the ones truly enhancing F508del-CFTR folding in a specific manner (for reviews, see [11, 12]). Moreover, because drug regulatory agencies require some indication of the MoA [92] for a compound to go from phase 0 (pre-clinical) into phase I, it is relevant to invest in drug-discovery assays that already provide this information.

Once lead compounds are identified by HT screening (HTS) of large libraries of small molecules, their respective mechanism of action (MoA) still has to be undermined, a process which is often time-consuming, and rate-limiting in their translation into the clinical setting. Strategies are thus required to quickly pinpoint the targets/pathways affected by such lead compounds resulting from HTS's. An alternative, as discussed below, is to use targets as the starting point for screening.

INNOVATIVE THERAPEUTIC STRATEGIES

Manipulation of the Proteostasis Network

"Proteostasis", or protein homeostasis (which means keeping the cellular proteome in a folded, functional-competent state) is achieved by an ensemble of proteins mostly composed of molecular chaperones, but also folding and proteolytic enzymes, and traffic factors ensuring correct protein intracellular compartmentalization [93]. Manipulation of this "proteostasis" network to achieve disease correction by controlling the chaperone machineries that maintain the folding status of the proteome has been recently proposed as a therapeutic strategy to diminish the global threshold at which non-native proteins are sent for degradation (reviewed in [93, 94]). Application of the "proteostasis" approach to CF therapy would imply allowing some F508del-CFTR protein to escape the tight ERQC control [95]. Likely, however, this may have substantial side-effects as the same "lower ERQC threshold" would apply to *all* misfolded proteins *en route* to in the secretory pathway which, under normal conditions, would be retained by the ERQC and sent for ERAD.

Indeed, ERAD is a very general "clearance" mechanism which is believed to have evolved to efficiently protect the cell from accumulation of abnormal proteins within the secretory pathway. Incompetent ERAD would cause irreversible cell damage, pathological states, premature aging and decreased endurance to environmental stresses. Although the proposers of therapeutic proteostasis claim that there is enough *room* to lower the "pass level" for protein conformers attempting to enter the secretory pathway [95], there is recent evidence that this concept may not hold. Indeed, in the case of retinitis pigmentosa, a retinal degeneration caused by mutations in the rhodopsin-1 gene and causing ER stress, it

was found by studying the fly model of this disease where similar mutations in the same gene trigger age-related retinal degeneration, that ERAD acts as a protective mechanism against retinal degeneration [96]. Moreover, these results suggest that manipulation of ERAD towards increasing, not decreasing, its stringency may also serve as a powerful therapeutic strategy against retinitis pigmentosa and probably also a number of diseases associated with ER stress.

Displacement of Molecular Chaperones/Factors

Another interesting therapeutic approach is "chaperone displacement", which has been shown to rescue F508del-CFTR to the cell membrane by saturating some molecular chaperones of the ERQC by using (misfolded) parts of CFTR as "decoys" [97]. A similar strategy, but using peptides containing the arginine-framed tripeptide (AFT) signals known to retain F508del-CFTR within the ER has also recently been shown to be successful in cell culture [98]. The latter is particular appealing since usage of peptides has recently proven successful in a phase II clinical trial, albeit for another disease [99]. The attractiveness of this approach is that, like proteostasis manipulation, it does not rely on the pre-identification of the key ERQC intervenients.

Although this displacement by competition does not necessarily imply complete specificity, there is some indication that more or less specific ERQC factors exist both for ER-Golgi transport [100, 101] and for ERAD [102]. Technically, however, such factors would not be called chaperones, which by definition are unspecific, and consequently the approach, would become "factor displacement". For CFTR, despite that multiple factors have been identified, including some not affecting overall secretory substrates (like the pro-degradative CHIP, targeting F508del-CFTR for ERAD [103]) strict specificity may not be guaranteed. Nevertheless, this approach is expected to be of more restricted scope than manoeuvring proteostasis.

So, the major difference between therapeutic proteostasis and chaperone/factor displacement approaches, although the real molecular mechanisms remain unidentified, seems to lie on the fact that the latter "saturates" the ERQC by "specific competition", thus diminishing to some extent the risk of general misconformer leakage. In other words, although both these strategies focus on widening the ERQC (and plausibly not so much on promoting F508del-CFTR intrinsic folding), molecular chaperone/factor displacement does so in a more or less *specific* way.

Targeting Mutant CFTR by *in silico* Small-Molecule Screens

Another very promising avenue to drug discovery aimed at correcting the basic defect in CF involves determination of the structural differences between wt-CFTR and its mutant variants namely, F508del-CFTR. The "holy grail" in the protein structure field is of course the high-resolution 3D-structure resolved by X-ray crystallography. But applying it to CFTR is a hard task because, like other members of the ABC transporter family, full-length CFTR (not to mention F508del-CFTR) is difficult to be obtained in significant yields as a pure protein so as to generate high-quality crystals. So, until these technical difficulties are solved, efforts were put to solving the wt- and F508del versions of NBD1 (where F508 is located). Surprisingly, the two structures were found to almost overlap [104, 105]. Nevertheless, the mutations used to solubilise F508del-NBD1 generated for this purpose were subsequently shown to rescue its trafficking defect *in vivo* [106], thus raising the possibility that the true structure of F508del-NBD1 may remain unresolved.

As alternatives to high-resolution structures, approaches such as cryo-electronic microscopy [107] and nuclear magnetic resonance (NMR) have been also explored in the CFTR field. Compelling NMR studies provided structural details for the mechanistic activation of CFTR by PKA-phosphorylation of the RD through loss of interaction with NBD1 [108, 109]. These results could also confirmed by computational models [110], thus indicating that the individual domains of CFTR largely contribute to self-chaperoning the full-length protein and that deletion of F508 diminishes this ability [111].

Protein modelling approaches have also been used to identify the structural differences between wt- and F508del-NBD1/CFTR, as an alternative /complementary approach. A recent molecular dynamics modelling study comparing the two versions of NBD1 has shown that the F508del domain has more conformational freedom and exposes more often its hydrophobic interior to the solution than wt-NBD1 [112]. Such increased dynamic flexibility may explain the recently reported five-fold higher affinity of F508del-CFTR for Hsp70 than wt-CFTR [86], the fact that the mutant is a better target for the ERQC as well as its diminished self-chaperoning ability [111]. Furthermore, major progress towards identification of the structural difference between wt and F508del-CFTR was achieved by a recent structural model of full-length CFTR, based on the atomic structure of the Sav1866 ABC transporter from *Staphylococcus aureus* [113]. This study identified a key intramolecular site of high potential to be repaired therapeutically between the surface of NBD1 involving the side chain of F508 and an intracellular loop (ICL4) in the second membrane-spanning domain (MSD2). Indeed, this NBD1-ICL4 site may be the putative binding site for F508del-CFTR correctors, while a previously proposed pocket located in the interface between NBD1 and NBD2 may be the binding site for CFTR channel potentiators [114]. Efforts to find small molecules that are able to fit into this structural pocket and to rescue the trafficking defect of F508del-CFTR are already underway.

Systems Biology Approaches

In the current post-genomic/ functional genomic era, research activities worldwide have started to focus on genome-wide, HT approaches to solve both mechanisms and therapies. One very relevant approach, while identifying the complete interactome of CFTR, demonstrated that down-regulation of a specific co-chaperone of Hsp90, Aha1 (activator of 90 kDa heat shock protein ATPase homolog1, AHSA1) partially rescued the processing defect of F508del-CFTR [115].

Another high-content study by Rotin and cols [116], overexpressed ~450 cDNAs fused to the halide-sensitive YFP marker in order to identify factors promoting rescue of F508del-CFTR to the plasma membrane of non-epithelial HEK 293 cells, in a variation of the previously described halide-permeability assay [117]. Among the 9 top hits resulting from this functional screen which were selected for further validation, only one (STAT1, Signal transducer and activator of transcription 1) could rescue F508del-CFTR to the cell surface above levels of corrector 4a. The drug Velcade (active compound proteasome inhibitor bortezomib, PS-341) was proposed in the study to act by a putative mechanism of action (not supported by evidence) involving Hsp70 and HspA4 (a non-validated screen hit). However, bortezomib is known to inhibit the "adaptive" unfolded protein response (UPR) by preventing ERAD. This is the mechanism by which bortezomib induces the pro-apoptotic UPR pathway, preferentially in myeloma cells as these produce high levels of immunoglobulin [118, 119] and the rationale for its usage as an anti-neoplastic agent. However, its use in other diseases, namely in CF is potentially dangerous given its pro-apoptotic properties.

In another recent study [120], suberoylanilide hydroxamic acid (SAHA), an histone deacetylase (HDAC) inhibitor and regulator of Hsp90 (a chaperone shown to regulate CFTR [77]), was described to restore the cell surface expression and channel activity of F508del-CFTR (to 28% of wt-CFTR) in human primary airway epithelial cells. By using a knock-down approach, this study revealed that HDAC7 plays a central role in rescue of F508del-CFTR by SAHA. However, although the therapeutic potential of this compound looks promising, the fact that it alters the expression of more than 1,000 genes, makes this approach unlikely to hit the clinical setting soon.

Additional experimental strategies such as high-content siRNA or cDNA overexpression screens using different assays, namely designed to specifically assess traffic, e.g., using fluorescent constructs coupled to automatic microscopy [121] are in progress in various CF-related laboratories and are likely to provide large-scale information on targets and pathway affecting CFTR traffic/function. However, the computational integration of these extensive in-progress data from biochemistry, molecular biology, genetics, biophysics, and proteomics, in a true "systems" approach to derive network models [122] is probably the only way for the complete comprehension of the factors that govern the relationships between genes and proteins involved in the pathophysiology of CF. Nevertheless, this approach has yet to demonstrate results, when applied to solve disease mechanisms.

Pre-clinical Assessment of "CFTR-Repairing" Molecules

Nevertheless, pre-clinical validation of this affluence of novel compounds in terms of their efficacy and MoA still requires substantial efforts, so that only the best are actually given to patients who are now starting to be in high demand for the several competing clinical trials in perspective. So far, efficacy testing on human bronchial epithelial (HBE) cells from CF patients is considered to be the "gold standard" for CFTR-repairing molecules going into clinical trial [36, 36] and a good correlation has been found between data collected for VX-770 in HBEs and clinical trial outcomes [37]. However, this good correlation regarding this potentiator compound is still insufficient to prove primary HBEs as the gold standard for compound validation. So, more data still need to be gathered for additional compounds, correctors namely, in order to demonstrate that indeed efficacy on primary HBEs correlates well with clinical efficacy. In particular, bioavailability of small molecules based exclusively on cell culture testing, may prevent good prediction of clinical efficacy. One example is given by the specific CFTR(inh)-172 [123] which is quite potent in inhibiting CFTR in primary HBE cultures [36, 124] but has failed to demonstrate the same efficacy in human native sweat glands [125] or human intestinal tissue (MDA lab, unpublished observations).

Furthermore, compounds like PTC-124, pre-validated for CF both in primary HBEs and mouse models [126] as well as for Duchenne/Becker muscular dystrophy (DMD/BMD) also in both primary human muscle cells and in mdx mice expressing dystrophin nonsense alleles [127] has shown controversial results in clinical trials for CF and has been suspended in a phase II DMD/BMD trial due to lack of demonstrated benefit [128].

Accordingly, pre-clinical validation directly on native human tissues *ex vivo*, such as rectal biopsies already commonly used for the diagnosis and prognosis of CF in several European centres [129], or even samples of explanted CF airways, becomes an attractive option to assess bioavailability /efficacy of compounds. This approach may complement those on primary HBEs to achieve a better prediction of compound clinical value in human individuals.

Animal models have provided valuable insights into various aspects of CF and should also perform an important role in what concerns pre-clinical validation of small molecules. However, since mouse models lacking functional CFTR do not develop the characteristic manifestations of human CF, [130-132], the value of murine animals to predict the outcome of CFTR-repairing compounds maybe somewhat limited. Moreover, given that human and murine CFTR exhibit different channel characteristics [133] and also different regulatory pathways operate in the murine and human tissues [134], it is predicted that efficacy of small molecules in mice may not translate into equivalent effectiveness in the human lung. Indeed, as above described for primary cultures, testing of the CFTR (inh)-172 compound in mice has also shown efficient inhibition [123] and yet the same effect fails to be observed in human tissues. This leads to think that prediction of clinical efficacy based on validation of CFTR-repairing compounds in mouse models may be of limited value.

Notwithstanding, better predictions and pre-clinical validation are expected from testing of small molecules in novel animal models for CF, in pigs namely, whose anatomy, biochemistry, physiology, size, lifespan and genetics are more similar than mice to those of humans [135]. However, pre-clinical testing for correctors in the forthcoming F508del/F508del CF pig model [136] may be hampered by the leaky processing of porcine F508del-CFTR, which is described to be even higher than murine F508del-CFTR [137]. An ideal animal CF model to test for efficacy of correctors of human 508del-CFTR, would thus be a *CFTR -/-*

pig engineered to express human (two copies) of the human F508del-CFTR gene.

CONCLUSION

In summary, while a few innovative in-progress approaches seek more mechanistic insight into CF disease and a more efficient track to drugs, several novel compounds emerged from HTS as promising effective drugs against the basic defect in CF. Moreover, the first rationale therapies for CF relying on the understanding of the basic defect have started to hit the clinical setting. Based on the current "drug pipeline", these are expected to rise in numbers very soon.

Moreover, establishing good predictive pre-clinical assays and adequate therapy endpoints for "CFTR-repairing" therapies is indispensable to bring only the best compounds to the clinical setting and to assess their efficacy. To this end, the current diagnosis methods directly dependent on CFTR function (sweat Cl^- measurements, nasal potential difference or Cl^- secretion assessment in rectal biopsies) may be extremely helpful. With such a concerted action, it may be possible to achieve ~10% normal CFTR activity in the epithelia of CF patients, as generally believed to be sufficient to cure CF [70].

ACKNOWLEDGEMENTS

Work in the author's laboratory is supported by research grants in this area, namely, PIC/IC/83103/2007 and BioFiG funding (FCT/FEDER, Portugal/ European Union) and from European Union grant TargetScreen2 EU-FP6-2005-LH-7-037365.

ABBREVIATIONS

ABC = ATP-binding cassette (transporters) (transporters)

ASL = Airway surface liquid

CaCC = Ca^{2+}-activated Cl^- channels

CF = Cystic fibrosis

CFTR = Cystic fibrosis transmembrane conductance regulator

ENaC = Na^+ Epithelial channel

ER = Endoplasmic reticulum

ERAD = ER-associated degradation

ERQC = ER quality control

HBE = Human bronchial epithelial (cells)

HDAC = Histone deacetylase

HT = High-throughput

ICL = Intracellular loop

MoA = Mechanism of action

MSD = Membrane spanning domains

NBD = Nucleotide binding domain

NMR = Nuclear magnetic resonance

PTC = Premature termination codon

RD = Regulatory domain

Ub = Ubiquitin

UPR = Unfolded protein response

UPP = Ub-proteasomal pathway.

REFERENCES

[1] Storey S, Wald G. Novel agents in cystic fibrosis. Nat Rev Drug Discov 2008; 7: 555-6.

[2] Rowe SM, Miller S, Sorscher EJ. Cystic fibrosis. N Engl J Med 2005; 352: 1992-2001.

[3] Accurso FJ. Update in cystic fibrosis 2005. Am J Respir Crit Care Med 2006; 173: 944-7.

[4] Kerem B, Kerem E. The molecular basis for disease variability in cystic fibrosis. Eur J Hum Genet 1996; 4: 65-73.

[5] De Boeck K, Wilschanski M, Castellani C, *et al.* Cystic fibrosis: terminology and diagnostic algorithms. Thorax 2006; 61: 627-35.

[6] Riordan JR, Rommens JM, Kerem B, *et al.* Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 1989; 245: 1066-73.

[7] Welsh MJ, Ramsey BW, Accurso FJ, *et al.* Cystic Fibrosis. Scriver CR, Beaudet AL, Sly WS, Valle D, Eds. The Metabolic Basis of Inherited Disease. 8th: 5121-5188. 2001. New York, McGraw-Hill.

[8] Knowles MR, Stutts MJ, Spock A, *et al.* Abnormal ion permeation through cystic fibrosis respiratory epithelium. Science 1983; 221: 1067-70.

[9] Mall M, Bleich M, Greger R, *et al.* The amiloride-inhibitable Na+ conductance is reduced by the cystic fibrosis transmembrane conductance regulator in normal but not in cystic fibrosis airways. J Clin Invest 1998; 102: 15-21.

[10] Boucher RC. Regulation of airway surface liquid volume by human airway epithelia. Pflugers Arch 2003; 445: 495-8.

[11] Amaral MD, Kunzelmann K. Molecular targeting of CFTR as a therapeutic approach to cystic fibrosis. Trends Pharmacol Sci 2007; 28: 334-241.

[12] Riordan JR. CFTR function and prospects for therapy. Annu Rev Biochem 2008; 77:701-26.: 701-26.

[13] The CFTR Mutation Database. http://www sickkids on ca/cftr 2009.

[14] Kerem B, Rommens JM, Buchanan JA, *et al.* Identification of the cystic fibrosis gene: genetic analysis. Science 1989; 245: 1073-80.

[15] Cheng SH, Gregory RJ, Marshall J, *et al.* Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell 1990; 63: 827-34.

[16] Collins FS. Cystic fibrosis: molecular biology and therapeutic implications. Science 1992; 256: 774-9.

[17] Bobadilla JL, Macek M, Jr., Fine JP, *et al.* Cystic fibrosis: a worldwide analysis of CFTR mutations--correlation with incidence data and application to screening. Hum Mutat 2002; 19: 575-606.

[18] The Molecular Genetic Epidemiology of Cystic Fibrosis. Report of a joint meeting of WHO/ECFTN/ICF(M)A/ECFS. Downloadable at: http://www.cfww.org/WHO_index.asp: World Health Organization; 2004.

[19] Kerem E. Mutation specific therapy in CF. Paediatr Respir Rev 2006; 7 Suppl 1: S166-S169.

[20] Noone PG, Knowles MR. 'CFTR-opathies': disease phenotypes associated with cystic fibrosis transmembrane regulator gene mutations. Respir Res 2001; 2: 328-32.

[21] Welsh MJ, Smith AE. Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. Cell 1993; 73: 1251-4.

[22] Zielenski J, Tsui LC. Cystic fibrosis: genotypic and phenotypic variations. Annu Rev Genet 1995; 29: 777-807.

[23] Amaral MD. CFTR and chaperones: processing and degradation. J Mol Neurosci 2004; 23: 41-8.

[24] Amaral MD, Pacheco P, Beck S, *et al.* Cystic fibrosis patients with the 3272-26A>G splicing mutation have milder disease than F508del homozygotes: a large European study. J Med Genet 2001; 38: 777-83.

[25] Highsmith WE, Burch LH, Zhou Z, *et al.* A novel mutation in the cystic fibrosis gene in patients with pulmonary disease but normal sweat chloride concentrations. N Engl J Med 1994; 331: 974-80.

[26] Amaral MD. Cystic Fibrosis-Translating Basic Science Knowledge into Therapies. US Respiratory Disease 2011, in press.
[27] Wilschanski M, Yahav Y, Yaacov Y, *et al.* Gentamicin-induced correction of CFTR function in patients with cystic fibrosis and CFTR stop mutations. N Engl J Med 2003; 349: 1433-41.
[28] Rowe SM, Clancy JP. Pharmaceuticals targeting nonsense mutations in genetic diseases: progress in development. BioDrugs 2009; 23: 165-74.
[29] Goodier JL, Mayer J. PTC124 for cystic fibrosis. Lancet 2009; 373: 1426-7.
[30] Nudelman I, Glikin D, Smolkin B, *et al.* Repairing faulty genes by aminoglycosides: Development of new derivatives of geneticin (G418) with enhanced suppression of diseases-causing nonsense mutations. Bioorg Med Chem 2010; [Epub ahead of print].
[31] Pedemonte N, Lukacs GL, Du K, *et al.* Small-molecule correctors of defective DeltaF508-CFTR cellular processing identified by high-throughput screening. J Clin Invest 2005; 115: 2564-71.
[32] Results from phase IIa clinical trial of VX-809. http://www.cff.org 2010.
[33] Illek B, Fischer H, Santos GF, *et al.* cAMP-independent activation of CFTR Cl channels by the tyrosine kinase inhibitor genistein. Am J Physiol 1995; 268: C886-93.
[34] Illek B, Zhang L, Lewis NC, *et al.* Defective function of the cystic fibrosis-causing missense mutation G551D is recovered by genistein. Am J Physiol 1999; 277: C833-9.
[35] Moran O, Zegarra-Moran O. A quantitative description of the activation and inhibition of CFTR by potentiators: Genistein. FEBS Lett 2005; 579: 3979-83.
[36] Van GF, Hadida S, Grootenhuis PD, *et al.* Rescue of CF airway epithelial cell function *in vitro* by a CFTR potentiator, VX-770. Proc Natl Acad Sci USA 2009; 106: 18825-30.
[37] Results from phase IIb clinical trial of VX-770. http://www.cff.org 2009.
[38] Kalin N, Claass A, Sommer M, *et al.* DeltaF508 CFTR protein expression in tissues from patients with cystic fibrosis. J Clin Invest 1999; 103: 1379-89.
[39] Penque D, Mendes F, Beck S, *et al.* Cystic fibrosis F508del patients have apically localized CFTR in a reduced number of airway cells. Lab Invest 2000; 80: 857-68.
[40] Dalemans W, Barbry P, Champigny G, *et al.* Altered chloride ion channel kinetics associated with the delta F508 cystic fibrosis mutation. Nature 1991; 354: 526-8.
[41] Wang F, Zeltwanger S, Hu S, *et al.* Deletion of phenylalanine 508 causes attenuated phosphorylation-dependent activation of CFTR chloride channels. J Physiol 2000; 524 Pt 3: 637-48.
[42] Du L, Gatti RA. Progress toward therapy with antisense-mediated splicing modulation. Curr Opin Mol Ther 2009; 11: 116-23.
[43] The CFTR2 Project (CFF). http://www genet sickkids on ca/cftr/app 2009.
[44] Wilschanski M, Yahav Y, Yaacov Y, *et al.* Gentamicin-induced correction of CFTR function in patients with cystic fibrosis and CFTR stop mutations. N Engl J Med 2003; 349: 1433-41.
[45] Clancy JP, Rowe SM, Bebok Z, *et al.* No detectable improvements in cystic fibrosis transmembrane conductance regulator by nasal aminoglycosides in patients with cystic fibrosis with stop mutations. Am J Respir Cell Mol Biol 2007; 37: 57-66.
[46] Clunes MT, Boucher RC. Front-runners for pharmacotherapeutic correction of the airway ion transport defect in cystic fibrosis. Curr Opin Pharmacol 2008; 8: 292-9.
[47] App EM, King M, Helfesrieder R, *et al.* Acute and long-term amiloride inhalation in cystic fibrosis lung disease. A rational approach to cystic fibrosis therapy. Am Rev Respir Dis 1990; 141: 605-12.
[48] Tomkiewicz RP, App EM, Zayas JG, *et al.* Amiloride inhalation therapy in cystic fibrosis. Influence on ion content, hydration, and rheology of sputum. Am Rev Respir Dis 1993; 148: 1002-7.
[49] Hirsh AJ, Zhang J, Zamurs A, *et al.* Pharmacological properties of N-(3,5-diamino-6-chloropyrazine-2-carbonyl)-N'-4-[4-(2,3-dihydroxypropoxy)phenyl] butyl-guanidine methanesulfonate (552-02), a novel epithelial sodium channel blocker with potential clinical efficacy for cystic fibrosis lung disease. J Pharmacol Exp Ther 2008; 325: 77-88.
[50] Boucher RC. Cystic fibrosis: a disease of vulnerability to airway surface dehydration. Trends Mol Med 2007; 13: 231-40.
[51] Hughey RP, Mueller GM, Bruns JB, *et al.* Maturation of the epithelial Na+ channel involves proteolytic processing of the alpha- and gamma-subunits. J Biol Chem 2003; 278: 37073-82.
[52] Hughey RP, Bruns JB, Kinlough CL, *et al.* Epithelial sodium channels are activated by furin-dependent proteolysis. J Biol Chem 2004; 279: 18111-4.
[53] Planes C, Caughey GH. Regulation of the epithelial Na+ channel by peptidases. Curr Top Dev Biol 2007; 78: 23-46.
[54] Sobczak K, Segal A, Bangel-Ruland N, *et al.* Specific inhibition of epithelial Na+ channels by antisense oligonucleotides for the treatment of Na+ hyperabsorption in cystic fibrosis. J Gene Med 2009; 11: 813-23.
[55] Caci E, Melani R, Pedemonte N, *et al.* Epithelial sodium channel inhibition in primary human bronchial epithelia by transfected siRNA. Am J Respir Cell Mol Biol 2009; 40: 211-6.
[56] Yueksekdag G, Drechsel M, Rossner M, *et al.* Repeated siRNA application is a precondition for successful mRNA gammaENaC knockdown in the murine airways. Eur J Pharm Biopharm 2010; [Epub ahead of print].
[57] Thomas M, Lu JJ, Chen J, *et al.* Non-viral siRNA delivery to the lung. Adv Drug Deliv Rev 2007; 59: 124-33.
[58] Yang YD, Cho H, Koo JY, *et al.* TMEM16A confers receptor-activated calcium-dependent chloride conductance. Nature 2008; 455: 1210-5.
[59] Caputo A, Caci E, Ferrera L, *et al.* TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. Science 2008; 322: 590-4.
[60] Schroeder BC, Cheng T, Jan YN, *et al.* Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. Cell 2008; 134: 1019-29.
[61] Kellerman D, Rossi MA, Engels J, *et al.* Denufosol: a review of studies with inhaled P2Y(2) agonists that led to Phase 3. Pulm Pharmacol Ther 2008; 21: 600-7.
[62] Teichgraber V, Ulrich M, Endlich N, *et al.* Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis. Nat Med 2008; 14: 382-91.
[63] Haggie PM, Verkman AS. Unimpaired lysosomal acidification in respiratory epithelial cells in cystic fibrosis. J Biol Chem 2009; 284: 7681-6.
[64] Haggie PM, Verkman AS. Defective organellar acidification as a cause of cystic fibrosis lung disease: reexamination of a recurring hypothesis. Am J Physiol Lung Cell Mol Physiol 2009; 296: L859-L867.
[65] Griesenbach U, Munkonge FM, Sumner-Jones S, *et al.* Assessment of CFTR function after gene transfer *in vitro* and *in vivo*. Methods Mol Biol 2008; 433: 229-42.
[66] Davies LA, McLachlan G, Sumner-Jones SG, *et al.* Enhanced lung gene expression after aerosol delivery of concentrated pDNA/PEI complexes. Mol Ther 2008; 16: 1283-90.
[67] Laner A, Goussard S, Ramalho AS, *et al.* Bacterial transfer of large functional genomic DNA into human cells. Gene Ther 2005; 12: 1559-72.
[68] Rocchi L, Braz C, Cattani S, *et al. E. coli* cloned CFTR loci relevant for human artificial chromosome therapy. Hum Gene Ther 2010.
[69] Denning GM, Ostedgaard LS, Welsh MJ. Abnormal localization of cystic fibrosis transmembrane conductance regulator in primary cultures of cystic fibrosis airway epithelia. J Cell Biol 1992; 118: 551-9.
[70] Amaral MD. Processing of CFTR: traversing the cellular maze--how much CFTR needs to go through to avoid cystic fibrosis? Pediatr Pulmonol 2005; 39: 479-91.
[71] Farinha CM, Amaral MD. Most F508del-CFTR is targeted to degradation at an early folding checkpoint and independently of calnexin. Mol Cell Biol 2005; 25: 5242-52.
[72] Roxo-Rosa M, Xu Z, Schmidt A, *et al.* Revertant mutants G550E and 4RK rescue cystic fibrosis mutants in the first nucleotide-binding domain of CFTR by different mechanisms. Proc Natl Acad Sci USA 2006; 103: 17891-6.
[73] Yang Y, Janich S, Cohn JA, *et al.* The common variant of cystic fibrosis transmembrane conductance regulator is recognized by hsp70 and degraded in a pre-Golgi nonlysosomal compartment. Proc Natl Acad Sci USA 1993; 90: 9480-4.
[74] Jiang C, Fang SL, Xiao YF, *et al.* Partial restoration of cAMP-stimulated CFTR chloride channel activity in DeltaF508 cells by deoxyspergualin. Am J Physiol 1998; 275: C171-8.

[75] Sugawara A, Torigoe T, Tamura Y, *et al.* Polyamine compound deoxyspergualin inhibits heat shock protein-induced activation of immature dendritic cells. Cell Stress Chaperones 2009; 14: 133-9.

[76] Farinha CM, Nogueira P, Mendes F, *et al.* The human DnaJ homologue (Hdj)-1/heat-shock protein (Hsp) 40 co-chaperone is required for the *in vivo* stabilization of the cystic fibrosis transmembrane conductance regulator by Hsp70. Biochem J 2002; 366: 797-806.

[77] Loo MA, Jensen TJ, Cui L, *et al.* Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome. EMBO J 1998; 17: 6879-87.

[78] Chang XB, Mengos A, Hou YX, *et al.* Role of N-linked oligosaccharides in the biosynthetic processing of the cystic fibrosis membrane conductance regulator. J Cell Sci 2008; 121: 2814-23.

[79] Rosser MF, Grove DE, Chen L, *et al.* Assembly and misassembly of cystic fibrosis transmembrane conductance regulator: folding defects caused by deletion of F508 occur before and after the calnexin-dependent association of membrane spanning domain (MSD) 1 and MSD2. Mol Biol Cell 2008; 19: 4570-9.

[80] Glozman R, Okiyoneda T, Mulvihill CM, *et al.* N-glycans are direct determinants of CFTR folding and stability in secretory and endocytic membrane traffic. J Cell Biol 2009; 184: 847-62.

[81] Egan ME, Glockner-Pagel J, Ambrose C, *et al.* Calcium-pump inhibitors induce functional surface expression of Delta F508-CFTR protein in cystic fibrosis epithelial cells. Nat Med 2002; 8: 485-92.

[82] Egan ME, Pearson M, Weiner SA, *et al.* Curcumin, a major constituent of turmeric, corrects cystic fibrosis defects. Science 2004; 304: 600-2.

[83] Norez C, Antigny F, Becq F, *et al.* Maintaining low Ca^{2+} level in the endoplasmic reticulum restores abnormal endogenous F508del-CFTR trafficking in airway epithelial cells. Traffic 2006; 7: 562-73.

[84] Norez C, Noel S, Wilke M, *et al.* Rescue of functional delF508-CFTR channels in cystic fibrosis epithelial cells by the alpha-glucosidase inhibitor miglustat. FEBS Lett 2006; 580: 2081-6.

[85] Cox T, Lachmann R, Hollak C, *et al.* Novel oral treatment of Gaucher's disease with N-butyldeoxynojirimycin (OGT 918) to decrease substrate biosynthesis. Lancet 2000; 355: 1481-5.

[86] Scott-Ward TS, Amaral MD. Deletion of Phe508 in the first nucleotide-binding domain of the cystic fibrosis transmembrane conductance regulator increases its affinity for the heat shock cognate 70 chaperone. FEBS J 2009; 276: 7097-109.

[87] Hohfeld J, Cyr DM, Patterson C. From the cradle to the grave: molecular chaperones that may choose between folding and degradation. EMBO Rep 2001; 2: 885-90.

[88] Cui L, Aleksandrov L, Hou YX, *et al.* The role of cystic fibrosis transmembrane conductance regulator phenylalanine 508 side chain in ion channel gating. J Physiol 2006; 572: 347-58.

[89] Sharma M, Benharouga M, Hu W, *et al.* Conformational and temperature-sensitive stability defects of the delta F508 cystic fibrosis transmembrane conductance regulator in post-endoplasmic reticulum compartments. J Biol Chem 2001; 276: 8942-50.

[90] Swiatecka-Urban A, Duhaime M, Coutermarsh B, *et al.* PDZ domain interaction controls the endocytic recycling of the cystic fibrosis transmembrane conductance regulator. J Biol Chem 2002; 277: 40099-105.

[91] Sharma M, Pampinella F, Nemes C, *et al.* Misfolding diverts CFTR from recycling to degradation: quality control at early endosomes. J Cell Biol 2004; 164: 923-33.

[92] Guidance for Industry, Investigators, and Reviewers. Exploratory IND Studies. US Department of Health and Human Services Food and Drug Administration.Center for Drug Evaluation and Research (CDER) 2006.

[93] Balch WE, Morimoto RI, Dillin A, *et al.* Adapting proteostasis for disease intervention. Science 2008; 319: 916-9.

[94] Powers ET, Morimoto RI, Dillin A, *et al.* Biological and chemical approaches to diseases of proteostasis deficiency. Annu Rev Biochem 2009; 78: 959-91.

[95] Hutt DM, Powers ET, Balch WE. The proteostasis boundary in misfolding diseases of membrane traffic. FEBS Lett 2009; 583: 2639-46.

[96] Kang MJ, Ryoo HD. Suppression of retinal degeneration in Drosophila by stimulation of ER-associated degradation. Proc Natl Acad Sci USA 2009; 106: 17043-8.

[97] Sun F, Mi Z, Condliffe SB, *et al.* Chaperone displacement from mutant cystic fibrosis transmembrane conductance regulator restores its function in human airway epithelia. FASEB J 2008; 22: 3255-63.

[98] Kim CP, Huan LJ, Gagnon S, *et al.* Functional rescue of DeltaF508-CFTR by peptides designed to mimic sorting motifs. Chem Biol 2009; 16: 520-30.

[99] Bates E, Bode C, Costa M, *et al.* Intracoronary KAI-9803 as an adjunct to primary percutaneous coronary intervention for acute ST-segment elevation myocardial infarction. Circulation 2008; 117: 886-96.

[100] Saito K, Chen M, Bard F, *et al.* TANGO1 facilitates cargo loading at endoplasmic reticulum exit sites. Cell 2009; 136: 891-902.

[101] Spang A. On vesicle formation and tethering in the ER-Golgi shuttle. Curr Opin Cell Biol 2009; 21: 531-6.

[102] Brodsky JL, Wojcikiewicz RJ. Substrate-specific mediators of ER associated degradation (ERAD). Curr Opin Cell Biol 2009; 21: 516-21.

[103] Younger JM, Ren HY, Chen L, *et al.* A foldable CFTR{Delta} F508 biogenic intermediate accumulates upon inhibition of the Hsc70-CHIP E3 ubiquitin ligase. J Cell Biol 2004; 167: 1075-85.

[104] Lewis HA, Buchanan SG, Burley SK, *et al.* Structure of nucleotide-binding domain 1 of the cystic fibrosis transmembrane conductance regulator. EMBO J 2004; 23: 282-93.

[105] Lewis HA, Zhao X, Wang C, *et al.* Impact of the deltaF508 mutation in first nucleotide-binding domain of human cystic fibrosis transmembrane conductance regulator on domain folding and structure. J Biol Chem 2005; 280: 1346-53.

[106] Pissarra L, Farinha C, Schmidt A, *et al.* The Effect of F508del-NBD1 solubilizing mutations on processing of full-length CFTR. Pediatr Pulmonol 2006; 29S: Abs 47, 224-5.

[107] Ford RC, Holzenburg A. Electron crystallography of biomolecules: mysterious membranes and missing cones. Trends Biochem Sci 2008; 33: 38-43.

[108] Baker JM, Hudson RP, Kanelis V, *et al.* CFTR regulatory region interacts with NBD1 predominantly via multiple transient helices. Nat Struct Mol Biol 2007; 14: 738-45.

[109] Kanelis V, Hudson RP, Thibodeau PH, Thomas PJ, Forman-Kay JD. NMR evidence for differential phosphorylation-dependent interactions in WT and DeltaF508 CFTR. EMBO J 2010; 29: 263-77.

[110] Hegedus T, Serohijos AW, Dokholyan NV, *et al.* Computational studies reveal phosphorylation-dependent changes in the unstructured R domain of CFTR. J Mol Biol 2008; 378: 1052-63.

[111] Serohijos AW, Hegedus T, Riordan JR, *et al.* Diminished self-chaperoning activity of the DeltaF508 mutant of CFTR results in protein misfolding. PLoS Comput Biol 2008; 4: e1000008.

[112] Wieczorek G, Zielenkiewicz P. DeltaF508 mutation increases conformational flexibility of CFTR protein. J Cyst Fibros 2008; 7: 295-300.

[113] Serohijos AW, Hegedus T, Aleksandrov AA, *et al.* Phenylalanine-508 mediates a cytoplasmic-membrane domain contact in the CFTR 3D structure crucial to assembly and channel function. Proc Natl Acad Sci USA 2008; 105: 3256-61.

[114] Moran O, Galietta LJ, Zegarra-Moran O. Binding site of activators of the cystic fibrosis transmembrane conductance regulator in the nucleotide binding domains. Cell Mol Life Sci 2005; 62: 446-60.

[115] Wang X, Venable J, LaPointe P, *et al.* Hsp90 cochaperone Aha1 downregulation rescues misfolding of CFTR in cystic fibrosis. Cell 2006; 127: 803-15.

[116] Trzcinska-Daneluti AM, Ly D, Huynh L, *et al.* High-content functional screen to identify proteins that correct F508del-CFTR function. Mol Cell Proteom 2009; 8: 780-90.

[117] Galietta LJ, Haggie PM, Verkman AS. Green fluorescent protein-based halide indicators with improved chloride and iodide affinities. FEBS Lett 2001; 499: 220-4.

[118] Lee AH, Iwakoshi NN, Anderson KC, *et al.* Proteasome inhibitors disrupt the unfolded protein response in myeloma cells. Proc Natl Acad Sci USA 2003; 100: 9946-51.

[119] Meister S, Schubert U, Neubert K, *et al.* Extensive immunoglobulin production sensitizes myeloma cells for proteasome inhibition. Cancer Res 2007; 67: 1783-92.

[120] Hutt DM, Herman D, Rodrigues AP, *et al.* Reduced histone deacetylase 7 activity restores function to misfolded CFTR in cystic fibrosis. Nat Chem Biol 2010; 6: 25-33.

[121] Pepperkok R, Ellenberg J. High-throughput fluorescence microscopy for systems biology. Nat Rev Mol Cell Biol 2006; 7: 690-6.
[122] Bansal M, Belcastro V, Ambesi-Impiombato A, *et al.* How to infer gene networks from expression profiles. Mol Syst Biol 2007; 3: 78.
[123] Ma T, Thiagarajah JR, Yang H, *et al.* Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. J Clin Invest 2002; 110: 1651-8.
[124] Fulcher ML, Gabriel SE, Olsen JC, *et al.* Novel human bronchial epithelial cell lines for cystic fibrosis research. Am J Physiol Lung Cell Mol Physiol 2009; 296: L82-L91.
[125] Wang XF, Reddy MM, Quinton PM. Effects of a new cystic fibrosis transmembrane conductance regulator inhibitor on Cl- conductance in human sweat ducts. Exp Physiol 2004; 89: 417-25.
[126] Du M, Liu X, Welch EM, *et al.* PTC124 is an orally bioavailable compound that promotes suppression of the human CFTR-G542X nonsense allele in a CF mouse model. Proc Natl Acad Sci USA 2008; 105: 2064-9.
[127] Welch EM, Barton ER, Zhuo J, *et al.* PTC124 targets genetic disorders caused by nonsense mutations. Nature 2007; 447: 87-91.
[128] Study of Ataluren (PTC124®) in Nonambulatory Patients With Nonsense-Mutation-Mediated Duchenne/Becker Muscular Dystrophy (nmDMD/BMD). http://clinicaltrials.gov/ct2/show/NCT01009294. 2010.
[129] Hirtz S, Gonska T, Seydewitz HH, *et al.* CFTR Cl- channel function in native human colon correlates with the genotype and phenotype in cystic fibrosis. Gastroenterology 2004; 127: 1085-95.
[130] Snouwaert JN, Brigman KK, Latour AM, *et al.* An animal model for cystic fibrosis made by gene targeting. Science 1992; 257: 1083-8.
[131] O'Neal WK, Hasty P, McCray PB, Jr., *et al.* A severe phenotype in mice with a duplication of exon 3 in the cystic fibrosis locus. Hum Mol Genet 1993; 2: 1561-9.
[132] van Doorninck JH, French PJ, Verbeek E, *et al.* A mouse model for the cystic fibrosis delta F508 mutation. EMBO J 1995; 14: 4403-11.
[133] Lansdell KA, Kidd JF, Delaney SJ, *et al.* Regulation of murine cystic fibrosis transmembrane conductance regulator Cl- channels expressed in Chinese hamster ovary cells. J Physiol 1998; 512: 751-64.
[134] Nadeau JH. Modifier genes in mice and humans. Nat Rev Genet 2001; 2: 165-74.
[135] Rogers CS, Stoltz DA, Meyerholz DK, *et al.* Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Science 2008; 321: 1837-41.
[136] Rogers CS, Hao Y, Rokhlina T, *et al.* Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. J Clin Invest 2008; 118: 1571-7.
[137] Ostedgaard LS, Rogers CS, Dong Q, *et al.* Processing and function of CFTR-DeltaF508 are species-dependent. Proc Natl Acad Sci USA 2007; 104: 15370-5.

Received: December 04, 2009 Revised: April 30, 2010 Accepted: April 30, 2010

PMID: 21039334

ABC Subfamily D Proteins and Very Long Chain Fatty Acid Metabolism as Novel Targets in Adrenoleukodystrophy

Masashi Morita[1], Nobuyuki Shimozawa[2], Yoshinori Kashiwayama[1], Yasuyuki Suzuki[3] and Tsuneo Imanaka[*,1]

[1]*Department of Biological Chemistry, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan*

[2]*Division of Genomic Research, Life Science Research Center, Gifu University Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan*

[3]*Medical Education Development Center, Gifu University Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan*

Abstract: Peroxisomes are involved in a variety of metabolic processes, including β-oxidation of fatty acids, especially very long chain fatty acids. Three peroxisomal ABC proteins belonging to subfamily D have been identified in mammalian peroxisomes that have an important role in fatty acid metabolism. ABCD1/ALDP and ABCD2/ALDRP are suggested to be involved in the transport of very long chain acyl-CoA, and ABCD3/PMP70 is involved in the transport of long chain acyl-CoA. ABCD1 is known to be responsible for X-linked adrenoleukodystrophy (X-ALD); an inborn error of peroxisomal β-oxidation of very long chain fatty acids. X-ALD is characterized biochemically by the accumulation of very long chain fatty acids in all tissues, including the brain white matter. Progressive demyelination of the central nervous system and adrenal dysfunction have been observed. The pharmacological up-regulation of peroxisomal β-oxidation of very long chain fatty acids and the suppression of fatty acid elongation are important aspects of an optimal therapeutic approach. Attractive targets for the treatment of X-ALD patients include the ABCD2 as well as elongase that is involved in the elongation of very long chain fatty acids. In addition, stabilization of mutant ABCD1 that has retained some of its function might be another approach, since most of the mutant ABCD1s with a missense mutation are degraded rapidly by proteasomes before or after targeting to peroxisomes. Protection of the central nervous system against oxidative damage is also important in order to delay the progress of disease. We summarize recent pharmaceutical studies and consider the potential for future X-ALD therapies.

Keywords: ABC protein, adrenoleukodystrophy, fatty acid β-oxidation, neurodegeneration, peroxisome, very long chain fatty acids.

INTRODUCTION

Peroxisomes are organelles bounded by a single membrane that are present in almost all eukaryotic cells. These organelles are involved in a variety of metabolic processes, including the β-oxidation of fatty acids, especially very long chain fatty acids (VLCFA), and the synthesis of ether phospholipids and bile acids in mammals [1]. These metabolic pathways require the transport of metabolites in and out of peroxisomes [2]. Recently it has become clear that the transport of such metabolites is facilitated by at least several different metabolic transporters. One of the transporter families is the ATP-binding cassette (ABC) protein. They are a superfamily of membrane-bound proteins whose structure is highly conserved from eubacteria to mammals and which catalyze the ATP-dependent transmembrane transport of a wide variety of substrates, including lipids.

To date, three ABC proteins classified into "subfamily D" have been identified in mammalian peroxisomes. These are adrenoleukodystrophy protein (ALDP/ABCD1), ALDP-related protein (ALDRP/ABCD2), and a 70-kDa peroxisomal membrane protein (PMP70/ABCD3) [3-8]. Dysfunction of ABCD1 is the cause of the human genetic disorder, X-linked adrenoleukodystrophy (X-ALD), which is characterized by an accumulation of VLCFA because of an impaired peroxisomal β-oxidation of VLCFA. VLCFA β-oxidation in X-ALD patient fibroblasts was restored by the expression of ABCD1 [9-11]. Likewise, the expression of ABCD2, which has a high sequence similarity to ABCD1, also restored VLCFA β-oxidation in X-ALD fibroblasts [12]. These data indicate that ABCD1 and ABCD2 are involved in the metabolic transport of VLCFA. ABCD3 is suggested to be involved in the metabolic transport of long chain fatty acids, since the overexpression of ABCD3 in CHO cells induced the β-oxidation of palmitic acid [13]. *Abcd3*(-/-) knockout mice exhibited abnormalities in peroxisomal metabolism of the bile acid intermediates, pristanic acid and phytanic acid, suggesting that ABCD3 is involved in the transport of bile acid intermediates and branched chain fatty acids (Jimenez-Sanchez *et al. Am J Hum Genet* 2000, meeting abstract). ABCD3-related protein (P70R/ABCD4) is also a member of ABC protein subfamily D [5, 14]. Recently, we found that ABCD4 is localized to the endoplasmic reticulum (ER), not to peroxisomes, but the function of ABCD4 still remains unknown [15].

*Address correspondence to this author at the Department of Biological Chemistry, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan; Tel: +81-76-434-7545; Fax: +81-76-434-7545; E-mail: imanaka@pha.u-toyama.ac.jp

1389-4501/11 $58.00+.00 **© 2011 Bentham Science Publishers Ltd.**

X-ALD is caused by mutations in the ABCD1 gene [3] and affected patients show progressive demyelination in the central nervous system (CNS), adrenal insufficiency, and testicular dysfunction as pathological characteristics. Severe forms of X-ALD are associated with inflammatory demyelinative lesions. Lymphocytes, reactive astrocytes and macrophages are suggested to be involved in the formation of these lesions. The pathological features of X-ALD may be accounted for by the overabundance of VLCFA, and the abnormal accumulation of VLCFA is the most likely culprit behind the initiation and progression of the disease. However, the molecular basis of the disease is not well understood.

The therapeutic options for X-ALD are limited at present. Lorenzo's oil, a 4:1 mixture of glycerol triolein and glycerol trierucate, normalizes VLCFA levels in the plasma of X-ALD patient, but it does not alter the clinical progress of patients with neurological symptoms [16, 17]. Hematopoietic stem cell transplantation (HSCT) is currently the only effective treatment for X-ALD patients with the childhood cerebral form. However, the availability of HSCT is limited by compatible donors, and HSCT carries a high mortality risk from complications. In addition, HSCT is known to be effective only for asymptomatic patients and patients with the early stage of cerebral demyelination [18]. Under such circumstances, alternative therapeutic approaches based on biochemical and pathological characteristics of the disease obviously need to be developed. The pharmacological induction of *ABCD2* gene expression, stabilization of mutant ABCD1 with proper functionality, stimulation of VLCFA β-oxidation, inhibition of elongase, which is involved in VLCFA synthesis, and suppression of inflammatory events in glial cells, are all potentially important strategies in the treatment of X-ALD.

STRUCTURE AND FUNCTION OF ABCD1

Structure of ABCD1

ABCD1 has the predicted structure of a half-size ABC protein with one transmembrane domain (TMD) and one nucleotide-binding domain (NBD). The hydropathy profile of human ABCD1 predicts that the amino terminal half of ABCD1 is hydrophobic with six transmembrane segments, and the COOH-terminal half is hydrophilic, having NBD (Fig. **1A**) [19]. Protease treatment of peroxisomes indicated that the NBD of ABCD1 is exposed to the cytosol [19]. As

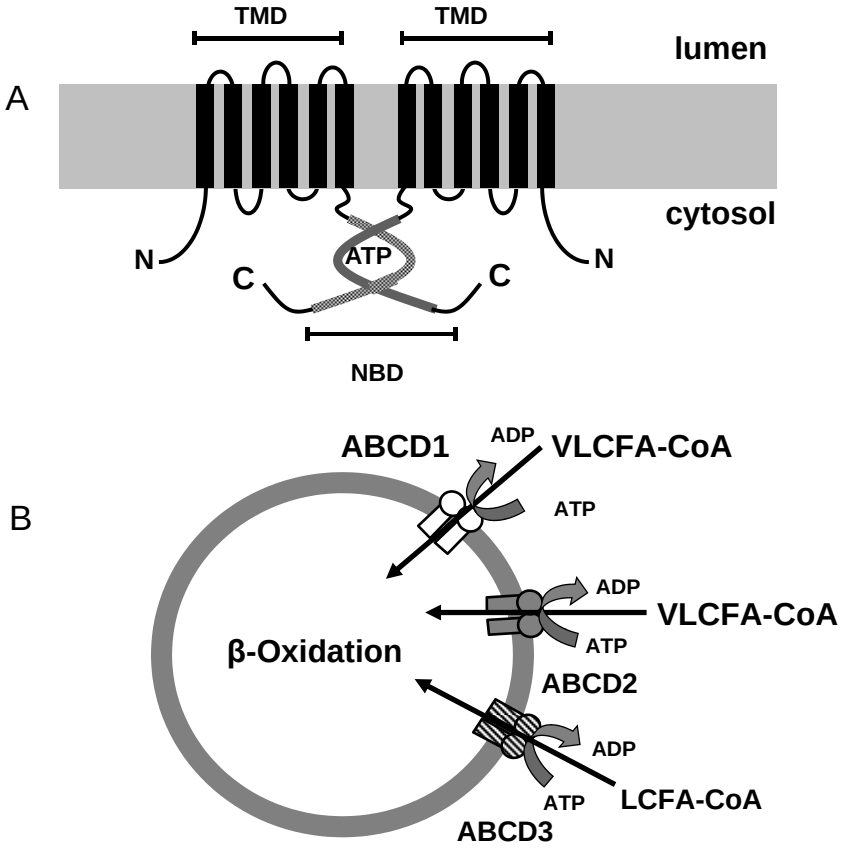


Fig. (1). The putative secondary structure of ABCD1 and function of peroxisomal ABC proteins. A, Six TMDs are located in the N-terminal half of the protein, and a NBD including the Walker A, B and ABC signature sequences are located in the C-terminal half of the protein. ABCD1 is a half-size ABC protein and predominantly exists as a homodimer. **B**, In mammals, three ABC proteins belonging to the D subfamily are known to exist in peroxisomes, ABCD1, ABCD2, and ABCD3. Among them, ABCD1 and ABCD2 have been suggested to be involved in the transport of very long chain acyl-CoA (VLCFA-CoA) from the cytoplasm into the peroxisomes. ABCD3 is thought to be involved in the transport of long chain acyl-CoA (LCFA-CoA).

for the ATP-binding and hydrolysis activities of ABCD1, Roerig *et al*. reported that the recombinant NBD of ABCD1 bound and hydrolyzed ATP [20]. Tanaka *et al.* also detected the ATP binding and hydrolysis activities of native ABCD1 in rat liver peroxisomes by photoaffinity labeling with 8-azido-[α-^{32}P]ATP and 8-azido-[γ-^{32}P]ATP [21]. On the other hand, the TMD of ABCD1 is proposed to be involved in substrate-recognition and to form a transport pathway across peroxisomal membranes. Guimarães *et al.* assessed the substrate-induced conformational alterations in ABCD1 with a protease-based assay, and found that long- and very long chain acyl-CoA increased the sensitivity of the NH_2-terminal 44-kDa fragment of ABCD1 to Factor Xa, and this acyl-CoA induced sensitivity was reversed by the presence of ATP-γS [22]. These findings suggest that the NH_2-terminal TMD of ABCD1 is involved in the recognition of these substrates and undergoes a conformational change upon ATP binding to the COOH-terminal NBD of ABCD1.

As most of the half-size ABC proteins identified to date dimerize to form a functional transporter, it has been suggested that the peroxisomal ABC proteins also need to assemble as homo- or heterodimers on the peroxisomal membranes to form a functional unit. As for the quaternary structures of peroxisomal ABC proteins, Liu *et al*. were the first to show the occurrence of homo- as well as heterodimeric interactions among ABCD1, ABCD2, and ABCD3 by using a yeast two-hybrid system and co-immunoprecipitation experiments [23]. We also showed by means of co-immunoprecipitation studies that ABCD1 forms a stable complex with ABCD3 and certain peroxisomal proteins on rat liver peroxisomal membranes [21]. On the other hand, Guimarães *et al*. reported that mouse liver ABCD1 was a mostly homomeric protein assembly, based on sucrose density gradient analysis and immunoprecipitation experiments with digitonin-solubilized mouse liver peroxisomes [24]. Furthermore, FRET microscopy experiments in intact living cells demonstrated that ABCD1 predominately forms a homodimer, although ABCD1 can form a heterodimer with ABCD3 [25]. These data suggest that ABCD1 mainly exists as a homodimer in mammalian peroxisomal membranes, although ABCD1 can form a complex with ABCD2 and ABCD3, and is involved in the ATP-utilizing transport of CoA derivatives of VLCFA.

Function of ABCD1

The biochemical hallmark of X-ALD is an impaired oxidation and accumulation of saturated VLCFA in cerebral white matter, adrenal glands, fibroblasts, and plasma [26]. Furthermore, *Abcd1*(-/-) mice also displayed the accumulation of VLCFA in tissues [27-29]. Although the precise mechanism by which ABCD1 is involved in fatty acid metabolism still remains to be elucidated, these pathognomonic characterizations of X-ALD allow us to deduce the function of ABCD1 to be a very long chain acyl-CoA transporter on peroxisomal membranes. Transfection of *ABCD1* cDNA into X-ALD skin fibroblasts restored the β-oxidation of lingoceric acid, and consequently, the VLCFA content returned to normal in the fibroblasts [9-11]. The expression levels of ABCD1 were correlated to VLCFA β-oxidation activities in primary and SV40T-transformed human skin fibroblasts [30]. Recently, van Roermund *et al.* showed that ABCD1 can function as a homodimer and is involved in the transport of a range of substrates including palmitic, oleic, behenic, and tetracosahexaenoic acid across the peroxisomal membranes by means of the expression of the human *ABCD1* cDNA in yeast *Saccharomyces cerevisiae* [31]. These findings strongly suggest that ABCD1 is involved in the uptake of activated VLCFAs into mammalian peroxisomes.

A variety of genetic and biochemical studies on ABCD1-related peroxisomal ABC proteins from other organisms have led to the same conclusion. For example, disruption of *PXA1* and/or *PXA2*, the only two peroxisomal ABC proteins known in *Saccharomyces cerevisiae*, resulted in impaired growth of these mutants on oleic acid as a sole carbon source, and a reduced ability to oxidize oleate [32-34]. Furthermore, Verleur *et al.* showed that Pxa2p is directly responsible for the ATP-dependent transport of long-chain acyl-CoA across peroxisomal membranes by using a semi-intact yeast cell system [35]. Recently, a similar role was proposed for a plant ABCD1 homologue, as well. In *Arabidopsis thaliana* the gene known variously as *PXA1*, *PED3*, or *CTS* encodes a full-size ABC protein mainly referred to as COMATOSE. Both halves of COMATOSE showed significant sequence identity to the human ABCD1. Zolman *et al.* reported that the *Arabidopsis PXA1* mutant grew slowly compared with wild type, with smaller rosettes, fewer leaves, and shorter inflorescence stems under hormone-free medium with auxin indole-3-butyric acid (IBA) [36]. IBA is converted to the more active auxin indole-3-acetic acid by peroxisomal β-oxidation, suggesting that the *PXA1* mutant has a defect in the import of IBA into peroxisomes. Recently, the loss of *CTS* in *Arabidopsis thaliana* exhibited a severe deficit in the breakdown of lipid bodies in germinated cotyledons [37]. In the *CTS* mutant, C20 and C22 acyl-CoA, which are predominantly derived from triacylglycerol, accumulated in seeds and seedlings. Furthermore, the *Arabidopsis PXA1* mutant under prolonged dark conditions exhibited an accumulation of free fatty acids, including palmitic acid, 7,10,13-hexadecatrienoic acid, and, α-linolenic acid, in mature leaves [38]. These findings also indicate that ABCD1 is involved in the uptake of activated VLCFAs across peroxisomal membranes.

MUTATION OF ABCD1 AND ADRENOLEUKODYSTROPHY

Mutations in ABCD1

The gene that is defective in X-ALD was mapped to Xq28 [39] and isolated and cloned by Aubourg and his colleagues [3]. The gene referred to as *ABCD1* is composed of 10 exons, and it codes for an mRNA of 4.3 kb, with a protein of 745 amino acids that is referred to as ABCD1. Thus far, more than 500 mutations widely distributed over the *ABCD1* gene have been identified (http://www.x-ald.nl). Missense mutations comprise ~60% of all of the mutations. The mutations have been found throughout the entire gene, although there is a clustering of mutations in the NH_2-terminal half of ABCD1, including TMD1-6, loop1-5 (40%) and NBD (30%) [40]. We have been analyzing the mutations of ABCD1 in Japanese probands with X-ALD and their families, and identified 55 mutations in 63 Japanese X-ALD kindreds, which included 35 missense mutations, 5 nonsense

mutations, 7 frame shift mutations, 3 amino acids deletions, 2 exon skip mutations and 3 large deletions

Among the missense mutation in X-ALD patients, it has been reported that ~50% of mutant ABCD1s were not detected and ~15% of them were reduced in amount in X-ALD fibroblasts, based on immunofluorescent or immunoblot analysis. Recently, we showed that some mutant ABCD1s were degraded by proteasomes or additional protease(s) before or after transport to peroxisomes [41].

Clinical Symptoms

There are various clinical phenotypes, such as the childhood cerebral form with cerebral demyelination and childhood onset (CCALD), the adolescent cerebral form (AdoCALD), the adult cerebral form (ACALD), adrenomyeloneuropathy (AMN) with axonopathy of the pyramidal and somatosensory tracts along with peripheral neuropathy, the olivo-ponto-cerebellar form (OPC), and Addison's disease alone [42]. We performed a retrospective nation-wide epidemiological survey of X-ALD in Japan during the 1990s [43]. However, no consistent correlation between phenotype and either specific mutations or ABCD1 expression was documented. The existence of modifier genes has been postulated [44].

CCALD is the most common phenotype, and is characterized by a progression of intellectual, psychological, visual and gait disturbances which first appear during the period school age. Patients are often misdiagnosed as having attention-deficit hyperactivity disorder, psychological problems, or either ophthalmic or ear abnormalities at the onset of the initial symptoms. Therefore, quite a number of patients are not diagnosed until further symptoms manifest, such as seizures, gait disturbances and/or other neurological symptoms. Brain MRI findings characteristically show an enhanced T2 signal, even at the early stage of the disease, suggesting extensive demyelination has occurred. It is hypothesized that the demyelination is associated with the cerebral inflammation. The prognosis is generally very poor and patients are considered likely to die within a few years, although recently good general care has apparently improved this unfavorable prognosis.

AdoCALD is similar to CCALD, but the appearance of symptoms occurs later than in CCALD, and develop more slowly. The age of onset is 11-21 years. ACALD is characterized by a slower progression of psychological symptoms, is more common in Japan than in Western countries [43], and is sometimes misdiagnosed as dementia or a psychological disorder. Most AMN patients manifest slowly progressive gait disturbances as the initial symptom, while sensory and autonomic disturbances occur in some patients. The mean age of onset of AMN was reported to be 30.2 (13-51) years in Japan [43], and about half of these patients exhibited cerebral involvement approximately 10 years after onset. OPC is characterized by cerebellar ataxia and pyramidal tract involvement, manifest gait disturbance as the initial symptom, and cerebellar ataxia which becomes evident several months to 1 year after onset. Approximately half of all OPC patients also display cerebral involvement, with concomitant symptoms such as intellectual and psychological problems [45]. This OPC form has been reported predominantly in Japan [46]. Patients with Addison's disease alone manifest adrenal insufficiency, including unexplained vomiting and weakness or coma, with first appearance occurring somewhere between childhood and adulthood.

Pre- or asymptomatic patients will progress to the various phenotypes described above between 3 and 50 years old. Therefore, it is important to provide information to patients and family members by means of genetic counseling. Diagnosis of pre-symptomatic boys before 3 years old, and long-term follow-up using subtle neuropsychological signs, brain MRI, electrophysiological investigation, adrenal function tests, and HSCT are of benefit. About half of the female carriers over the age of 40 years will develop mild to severe neurological symptoms caused by the spinal cord and peripheral nerve abnormalities, such as weakness and spasticity of the legs, impaired sensation of the lower limbs and autonomic disturbances, similar to AMN.

Pathogenesis of X-ALD

Mutations in the *ABCD1* gene result in increased VLCFA in tissues and body fluids of patients with X-ALD, and reduced VLCFA β-oxidation in peroxisomes. Impaired transport of very long chain acyl-CoA by ABCD1 explains, in part, the biochemical defects. Recently Ofman *et al.* have shown that a deficiency of ABCD1 raised cytosolic levels of very long chain acyl-CoAs in X-ALD fibroblasts and the substrates were then further elongated by the enzyme called elongation of very long chain fatty acids-1 (ELOVL1), which catalyzes the synthesis of both saturated (C26:0) and mono-unsaturated VLCFA (C26:1) [47]. In addition, knockdown of ELOVL1 reduced the elongation of C22:0 to C26:0 and lowered C26:0 levels in X-ALD fibroblasts, suggesting that ELOVL1, in addition to ABCD1, is important for the accumulation of VLCFA.

The mechanism by which the accumulation of VLCFA in the brain causes neurodegeneration, especially demyelination, remains obscure. However, the association of VLCFA accumulation with the immunoresponse is thought to be important (Fig. **2**). It has been demonstrated that VLCFA accumulation subsequently leads to a neuroinflammatory response, with the production of proinflmmatory cytokines by activated astrocytes and microgial cells together with demyelination and a loss of oligodendrocytes [48]. Direct toxicity of VLCFA has been demonstrated to result from an alteration of membrane fluidity in erythrocytes [49]. Recently, Hein *et al.* have shown a direct toxic effect of C26:0 on primary neurons and glial cells, especially oligodendrocytes, via mitochondrial dysfunction and Ca^{2+} deregulation [50]. The presence of less mature myelin in weaning and postweaning rats treated with hexacosanoic acid [51], suggests that an increase of VLCFA may lead to myelin instability, followed by an inflammatory or immune-mediated process, and therefore contribute, at least in part, to the loss of oligodendrocytes observed in the plaques of X-ALD brains. Moreover, the down-regulation of peroxisomal VLCFA β-oxidation may lead to a decrease of docosahexaenoic acid (DHA) synthesis. DHA is not directly incurporated into the myelin sheath, but its presence is crucial for oligodendrocyte maturation and the formation of mature myelin. The complex lipids of VLCFAs or VLCFA-phospholipids could serve as an antigen recognized by the

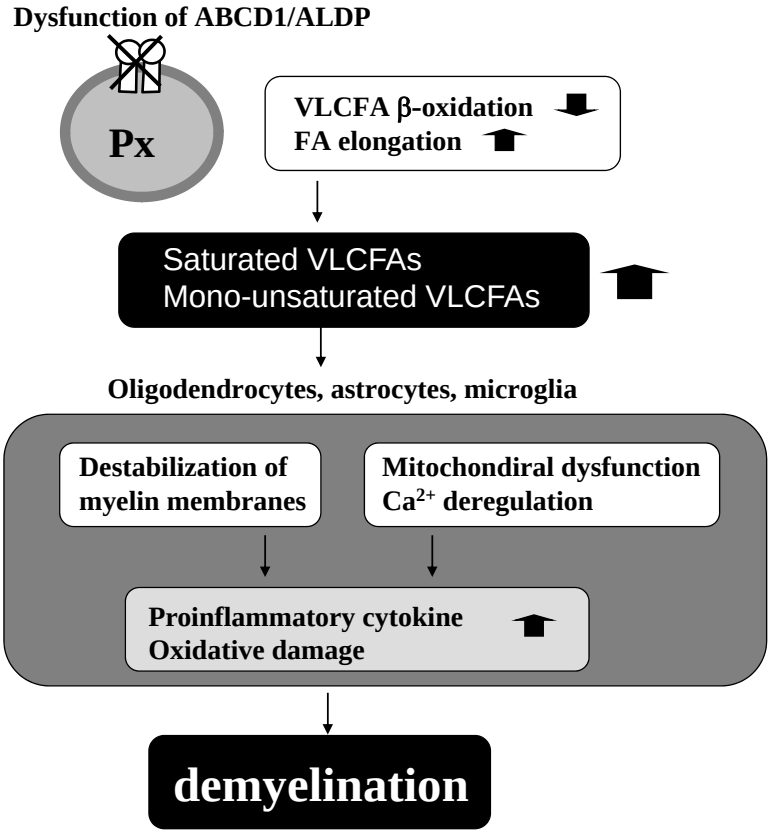


Fig. (2). A putative mechanism of neurodegeneration in X-ALD. In the CNS, abnormal accumulation of VLCFAs, which is caused by the dysfunction of ABCD1 followed by a reduction in peroxisomal VLCFA β-oxidation and/or an induction of fatty acid elongation, might result in the destabilization of myelin membranes and/or mitochondrial dysfunction. The myelin membrane fragments activate the astrocytes and/or microglia, which event leads to the inflammatory reaction. Proinflammatory cytokines are known to have negative affects on oligodendrocytes. Alternatively, an excess of VLCFA might adversely affect mitochondria, which would exacerbate oxidative stress in oligodendrocytes.

CD1 pathway, which would also be a plausible candidate to trigger inflammatory demyelination [52]. In addition, there is increasing evidence that oxidative stress contributes to the pathogenesis of the cerebral inflammatory phenotype, and possibly AMN. Gilg *et al*. demonstrated increased levels of inducible nitrous oxide synthase, and the presence of nitrosylated proteins in astrocytes and macrophages, in affected post-mortem tissue [53]. Powers *et al*. found convincing evidence of oxidative stress (increased levels of hemoxygenase 1 and manganese–superoxide dismutase) and oxidative damage from lipid peroxidation (4-hydroxynonal and malondialdehyde), as well as nitrosylated proteins, in the post-mortem brain tissue of four X-ALD patients with the cerebral inflammatory phenotype [54].

Diagnosis

The definitive diagnosis of X-ALD, as well as female carrier detection is achieved by demonstration of the biochemical defect along with mutation analysis of the *ABCD1* gene. An elevated VLCFA confirms the diagnosis of both X-ALD and the carrier, but there can be overlap of the C24:0/C22:0, C25:0/C22:0 and C26:0/C22:0 ranges between healthy controls and at least 10% of the carriers. Furthermore, 3 to 7 percent of patients with X-ALD are the result of a spontaneous mutation of the *ABCD1* gene, and thus the mothers of these patients are not carriers (hppt://www.x-ald.nl). For these reasons, mutation analysis of the *ABCD1* gene is necessary not only for carrier detection, but also for detecting a spontaneous mutation. Carrier detection is very important to identify pre-symptomatic patients and female carriers within their kindred. Newborn screening may be a potential method for widely identifying pre-symptomatic ALD patients and female carriers. Hubbard *et al.* have undertaken a mass screening pilot study by the detection of 1-hexacosanoyl-2-lyso-sn-3-glycerophosphoryl-choline (26:0-lyso-PC) using a combined liquid chromato-graphy-tandem mass spectrometry (LC-MS/MS) method [55].

Additionally, the establishment of a long-term follow-up system for these pre-symptomatic patients is needed, because we cannot at present predict the timing for HSCT so as to get beneficial effects or achieve a good clinical outcome. In our nationwide survey in Japan, approximately 30% of the X-ALD cases exhibited cerebral symptoms before the age of ten years, but nearly 50% of the patients had no obvious symptoms until they were twenty years old [43]. Thus, further break-throughs in diagnostic and therapeutic

approaches for X-ALD are keenly anticipated. In particular, the identification of predictive factors for the onset of brain involvement is of critical importance.

Therapeutic Approaches

The level of VLCFA does not predict the development of the disease state in X-ALD, and the exact link between abnormal VLCFA accumulation and the pathogenesis is still unclear at present. However, abnormal accumulation of VLCFA in brain is the most likely culprit for the initiation and progression of the disease.

Dietary therapy with Lorenzo's oil is now a commonly accepted treatment for X-ALD patients. Lorenzo's oil does not halt the progression of the cerebral phenotype X-ALD patients who were already symptomatic when the treatment was initiated. Recent reports, however, suggest that Lorenzo's oil might have demonstrated a preventive effect in asymptomatic boys whose brain MRI is normal [56, 57], and preliminary data from dietary therapy with Lorenzo's oil indicates that it seems to reduce the progression of pure AMN [58]. Golovko and Murphy reported that erucic acid (22:1n-9) can cross the blood-brain barrier, and is incurporated into cholesterol esters, triacylglycerols and phospholipids pools as either 22:1n-9 or its chain-shortened metabolites, 20:1n-9 and 18:1n-9, due to the chain shortening of 22:1n-9 [59]. It is thus possible that adverse structural changes in certain lipid classes in the X-ALD brain are attenuated by treatment with Lorenzo's oil, which might help prevent the disease onset. Elucidation of the precise mechanisms of Lorenzo's oil effect in the CNS could provide important clues to finding targets for the initiation of cerebral ALD, such as CCALD.

Lovastatin, an HMG-CoA inhibitor, is reportedly a potential therapeutic drug for X-ALD since it decreases plasma VLCFA in X-ALD patient [60]. However, in a comparable study using lovastatin and simvastatin, the VLCFA content could not be normalized in tissues and plasma of *Abcd1*(-/-) mice [61, 62]. It is hypothesized that lovastatin additionally may have a favorable effect, since it down-regulated the synthesis of NO in X-ALD lymphoblasts along with the decrease in VLCFA levels [63]. However, the

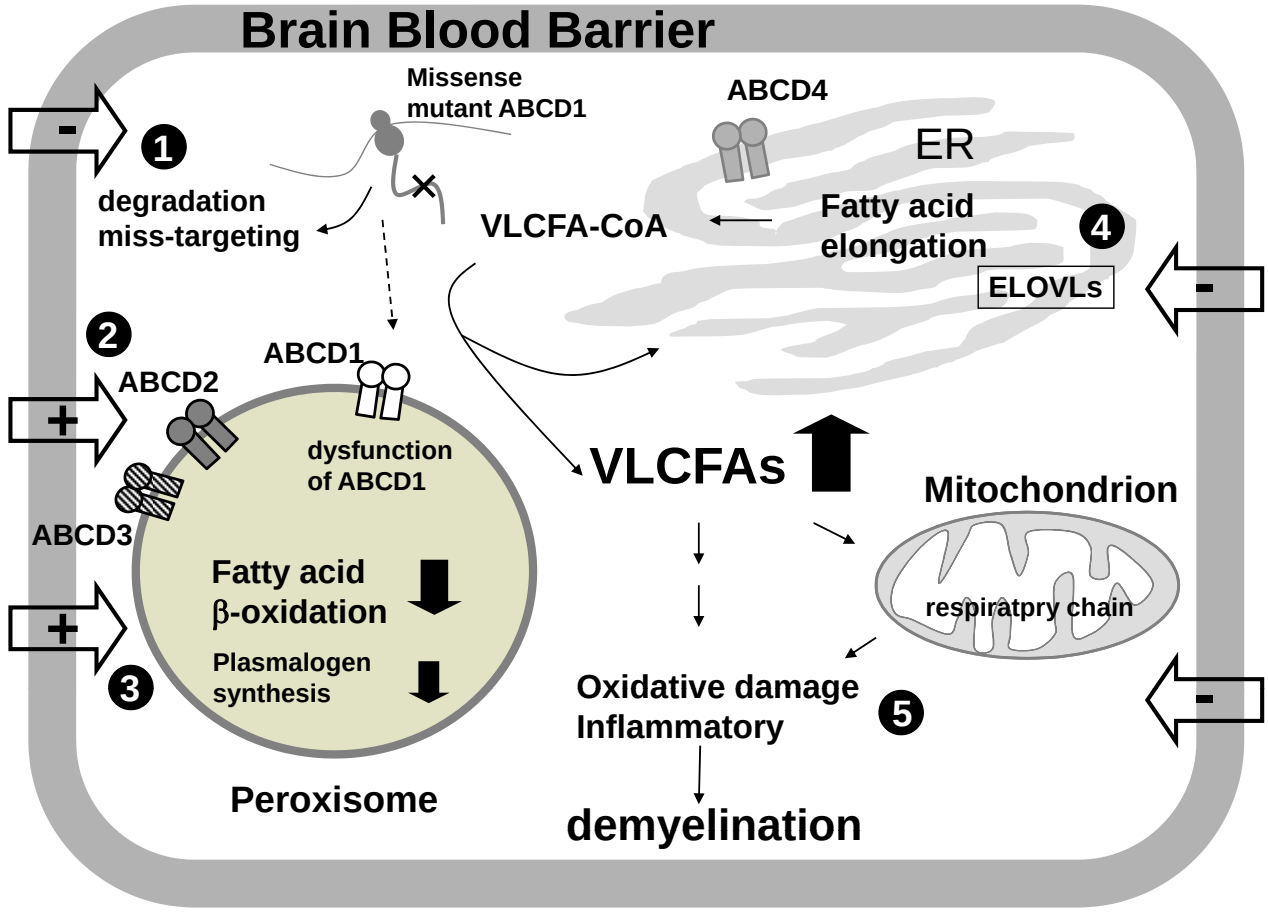


Fig. (3). Possible targets for X-ALD therapy. Mutation of ABCD1 leads to the dysfunction of ABCD1, which results in a decrease in VLCFA β-oxidation. The decrease in VLCFA β -oxidation leads to an increase in the VLCFA-CoA level, which is the substrate for fatty acid elongation in the ER. As a result, the VLCFA level is increased and the subsequent abnormal accumulation of VLCFAs results in oxidative damage. Stabilization and correct subcellular localization of missence mutants of ABCD1 with residual activity might be an effective way to recover the dysfunction of ABCD1 ❶. Induction of *ABCD2* expression ❷, stimulation of peroxisomal fatty acid β-oxidation ❸ or inhibition of microsomal fatty acid elongation ❹ are attractive approaches to reduce excess VLCFA levels. Attenuation of the dysregulated response to oxidative stress also has potential as an effective approach in X-ALD ❺. Chemical compounds that can pass through brain-blood barrier and act on these targets would be promising drugs for X-ALD therapy.

clinical efficacy of lovastatin has not yet been clarified. Very recently Engelen *et al.* demonstrated lovastatin leads only to a small decrease of plasma C24:0 and C26:0 in X-ALD patients by a nonspecific result of the decrease in the level of LDL cholesterol and they indicated that lovastatin should not be prescribed as a therapy to lower levels of VLCFA in patients with X-ALD [64]. In the following sections, we will focus our attention on other attractive X-ALD therapeutic targets over the past few years (Fig. **3**).

ABCD2 as a Target Molecule

Among the members of ABC protein subfamily D, ABCD2 shows the highest similarity (88%) to ABCD1. Overexpression of ABCD2 prevents the accumulation of saturated VLCFA in the adrenal gland and brain, and neurological signs of disease, which are observed in *Abcd1*(-/-) mice [65]. In addition, the X-ALD phenotype is independent of the *ABCD2* genotype, and *ABCD2* can be excluded as a modifier locus for clinical diversity in X-ALD [66]. Taken together, the pharmacological induction of expression is a reasonable therapeutic strategy for X-ALD.

ABCD2 transcription is regulated by nuclear factors such as the peroxisome proliferator-activated receptor (PPARα), retinoid X receptor (RXR), thyroid hormone receptor (TRβ) and sterol regulatory element (SRE) binding proteins (SREBP1a, SERBP1c and SREBP2). The SRE located in the *ABCD2* promoter overlaps with a direct repeat separated by 4 nucleotides (DR-4), suggesting cross talk between SREBPs and liver X receptor α (LXRα) or TRβ, which are known to be dimerized with RXRα [67]. Weinhofer *et al.* demonstrated that depression of the cholesterol content results in a decrease in the C26:0 level, because the expression of *ABCD2* gene is up-regulated via the activation of the SREBPs [68, 69]. In addition, the *ABCD2* promoter contains a functional thyroid hormone response element (TRE) by which thyroid hormone can induce the *ABCD2* gene both *in vitro* and *in vivo*. Recently, it was shown that the ligand-activated thyroid hormone receptors TRα and TRβ stimulate or derepress, respectively, the SREBP1-dependent induction of the *ABCD2* promoter [67]. These thyroid hormone receptors bind the SRE/DR-4 motif. Therefore, novel tissue-specific ligands for TRα, TRβ, or other DR-4 binding factors that interact with SREBP1, might enhance *ABCD2* expression in the brain. Recently, Genin *et al.* have reported that *ABCD2* gene is induced by the administration of halogen-free thyromimetics (GC-1 and CGS 23425) specific for TRβ receptors in *Abcd1*(-/-) mice. GC-1 was shown to be able to enter the brain and thus might be effective to induce gene expression, especially in oligodendrocytes. Therefore, these thyromimetics specific for TRβ may have the capacity to induce *ABCD2* genes in the brain [70] and therefore, could be attractive candidates for X-ALD therapy.

Dehydroepiandrosterone (DHEA), the most abundant steroid in humans, was reported to be a novel inducer of the *ABCD2* gene. Gueugnon *et al.* reported that DHEA directly induced *Abcd2* expression and that short-term treatment of mice with DHEA led to the induction of *Abcd2* expression independently of PPARα. Although the induction was only reportedly detected in the liver in their experiments, the long-term administration of DHEA may be beneficial for X-ALD patients [71].

Concerning the induction of *ABCD2*, 4-phenylbutyrate (4-PBA) and valproic acid, which are categorized as histone deacetylase (HDAC) inhibitors [72] , are potential candidates for the treatment of X-ALD. 4-PBA crosses the blood-brain barrier, and the administration of 4-PBA to *Abcd1*(-/-) mice resulted in the decrease in VLCFA level in the brain and adrenal glands via the induction of *ABCD2* expression or the activation of mitochondrial fatty acid β-oxidation [73]. However, this drug is very rapidly metabolized in the liver and has a very short half-life *in vivo*, indicating that the dosage required for a biological effect in humans makes it unpractical for clinical applications. Actually 4-PBA did not result in a decreased VLCFA levels in a small clinical trial with AMN patients. Therefore, structural analogs of 4-PBA with a longer half-life *in vivo* would be a more attractive candidate for X-ALD therapy [74]. Recently, Fourcade *et al.* have reported that valproic acid induced the expression of the *ABCD2* gene, reduced the level of monounsaturated VLCFA (C26:1) and induced antioxidant effects *in vivo* and *ex vivo* experiments [75]. Since valproic acid is known to be able to cross the blood-brain barrier and is already considered a very safe drug, valproic acid is a promising candidate for X-ALD.

The differences in the expression patterns and phenotypes of *Abcd1*(-/-) and *Abcd2*(-/-) mice suggest other specific roles for ABCD2 in lipid metabolism. Engelen *et al.* reported that cholesterol-deprivation led to the reduction of the C26:0 level along with the increase in *ABCD2* expression in X-ALD fibroblasts [76]. However, the depression of cholesterol resulted in an increased expression of stearoyl-CoA desaturase and increased mono-unsaturated VLCFA level, but did not show any increase in [1-^{14}C]C26:0 β-oxidation. Taken together, ABCD2 may be involved in other forms of metabolism than just C26:0 β-oxidation [76].

Leclercq *et al.* have reported that *ABCD2* expression in the liver is higher in n-3 polyunsaturated fatty acid (PUFA)-deficient rats than rats fed α-linoleic acid or docosahexaenoic acid, presumably because of feedback regulation. They speculated that ABCD2 is involved in the transport of specific classes of fatty acids related to PUFA metabolism into peroxisomes [77]. Fourcade *et al*. have also suggested that ABCD2 might play a role in the transport of DHA or docosapentaenoic acid (DPA) precursors (C24:5n-6 and C24:6n-3) and monounsaturated VLCFAs. They suggested that monounsaturated VLCFAs (C26:1) might be involved in oxidative damage to proteins [78]. These results are consistent with the report by Powers *et al.* that in *Abcd2*(-/-) mice, the oxidative stress in adrenal cells was grater than that in *Abcd1*(-/-) mice, suggesting that ABCD2 might be more important for the control of oxidative stress than ABCD1, at least in the adrenal glands [79].

Taken together, ABCD2 appears to have a central role in the metabolism of unsaturated rather than saturated VLCFAs, which pattern involves the synthesis of DHA and oxidative stress. Since DHA is correlated with the incidence of Alzheimer's disease, ABCD2 may serve as a therapeutic target in common human neurodegenerative disorders [78]. Although the precise role of ABCD2 remains to be elucidated, ABCD2 is a most promising target molecule for X-ALD therapy.

ABCD3 and ABCD4 as a Target Molecule

It has been reported that overexpression of ABCD3 restores VLCFA β-oxidation in X-ALD fibroblasts [10]. Furthermore, stimulation of ABCD3 through the activation of PPARα with fenofibrate overcame the peroxisomal β-oxidation defect in the liver of *Abcd1*(-/-) mice [12]. ABCD3 knockdown generated oxidative stress and pro-inflammatory cytokine production in C6 glial cells [80]. These results suggest that a correction of the biochemical defect in X-ALD should be possible by drug-induced overexpression or ectopic expression of ABCD3. Asheuer *et al.* studied the expression of *ABCD1-4* and two VLCFA synthetase genes (*VLCS* and *BG1*) involved in VLCFA metabolism to elucidate the mechanisms underlying the phenotypic variability of X-ALD. Among these genes, the expression of *ABCD4* and *BG1* tends to be decreased with the severity of the disease, acting early in the pathogenesis of X-ALD [81]. Therefore, ABCD3 and ABCD4 might be target molecules for X-ALD therapy. At present, however, approaches to induce ABCD3 and ABCD4 expression are not realistic since there is no report about induction of these expressions in mice and human brain.

Stability of Mutant ABCD1

It has been reported that ~70% of mutant ABCD1 with a missense mutation were either not detected or were reduced in X-ALD fibroblasts. We found that mutant ABCD1s with the missense mutation in the C-terminal half of ABCD1 were degraded by a protein quality control system associated with proteasomes, and ABCD1 mutants within the loop between TMD2 and 3 resulted in a deficiency in peroxisomal targeting [41]. Therefore, in the case where mutant ABCD1 has residual biological activities, stabilizing or correcting the subcellular localization of the mutant ABCD1 could restore its function. In cystic fibrosis and congenital nephritic syndrome of the Finnish type, defective trafficking of the missense mutants of transmembrane conductance regulator (CFTR) and nephrin, respectively, was rescued by a chemical chaperone, such as 4-PBA or flavonoids [82, 83]. Screening of small molecule libraries to stabilize or to correct the subcellular localization of functionally active ABCD1 mutants might be a beneficial approach for some X-ALD patients bearing a missense mutation. However, a very long way seems to be required to develop useful drugs from this point of view.

Stimulation of Peroxisomal β-Oxidation

Fatty acid β-oxidation activity in peroxisomes is thought to depend on the active transport of acyl-CoA across membranes and passive diffusion of free fatty acids within membranes. In the latter case, fatty acids are activated to acyl-CoA by very long chain acyl-CoA synthetases, such as ACSVL1 and ACSVL5, on the luminal side of peroxisomes. Pillia *et al.* demonstrated that free VLCFA diffuses rapidly through the lipid barrier [84]. Therefore, the activation of very long chain acyl-CoA synthetases in peroxisomes appears to be a target for X-ALD therapy. Recently we found that baicalein 5,6,7-trimethylether, a plant flavonoid, has the capacity to attenuate VLCFA metabolism in X-ALD fibroblasts [85]. This flavonoid activated peroxisomal fatty acid β-oxidation regardless of the carbon chain length. The activation appeared to be caused, at least in part, by the up-regulation of peroxisomal *ACSVL1*, but not *ABCD2*. Therefore, we speculated that baicalein 5,6,7-trimethylether activates peroxisomal fatty acid β-oxidation via an ABCD1-independent pathway [86]. The methoxy residues are important for the activity, suggesting the possibility that structural analogs may attain a greater potency. Further studies of flavonoids and chemically modified derivatives should help provide a new, metabolism-based therapeutic approach for X-ALD therapy.

Suppression of Fatty Acid Elongation

Saturated VLCFAs in the body are derived from both the diet and *de novo* synthesis. In contrast, excessive VLCFAs in X-ALD are largely derived from endogenous synthesis through fatty acid elongation in the ER. Reduction of saturated VLCFAs in plasma by Lorenzo's oil is presumably caused by competitive inhibition of the microsomal fatty acid elongation activity [87-89]. It is thus likely that the suppression of VLCFA synthesis provides a new approach for X-ALD.

The fatty acid elongation reaction in ER consists of 4 sequential reactions. In mammals, seven enzymes (ELOVL1-7) have been identified, which are responsible for the first and rate-limiting step in this reaction cycle [90]. High-density assay, a convenient assay method for measuring the elongation of VLCFA using a Unifilter-96 GF/C plate, revealed that ELOVL1, 3 and 6 preferentially elongated the saturated acyl-CoAs, while ELOVL2 and 5 elongated the unsaturated acyl-CoA [91]. Recently, ELOVL7 was shown to be involved in the elongation of saturated acyl-CoAs (C20:0 ~) [92].

Kemp and colleagues reported enhanced VLCFA elongation activity in X-ALD patient fibroblasts and speculated that they do not result from impaired peroxisomal β-oxidation alone, but also because of the additional effect of unchecked chain elongation [93]. As judged from the substrate specificity, ELOVL1 and ELOVL3 would be the most attractive candidate elongases for enhancing fatty acid elongation in X-ALD. Recently, it was reported that ELOVL1 expression is not increased in X-ALD fibroblasts, but the increased cytosolic very long chain acyl-CoA due to the reduction of peroxisomal fatty acid β-oxidation resulted in an increase in further elongation [47]. They also demonstrated that saturated VLCFA are synthesized via the concerted reaction of ELOVL6 and ELOVL1 and the silencing of ELOVL1 expression led to a decrease in C26:0 levels. The expression of *Elovl1* mRNA is ubiquitous in murine tissues, but potent expression is found in the myelinated parts of the CNS [90]. Although the expression of *ELOVL6* gene was reported to be regulated directly by SREBP1c [94], the transcriptional regulation of *ELOVL1* is still unclear. Down-regulation of *ELOVL1* and/or *ELOVL6* expression or identification of a specific inhibitor of ELOVL1 and/or ELOVL6 might be effective for the reduction of VLCFA.

Oxidative Damage

Several groups have proposed that oxidative stress might be involved in the pathogenesis of X-ALD. Although it is

not clear whether it is primary or secondary in the pathogenesis of X-ALD, the protection of the CNS from oxidative stress is suggested to be a useful therapeutic standpoint. Oxidative damage was demonstrated by the increased markers of lipoxidative damage in post mortem brain samples from cerebral X-ALD patients [54]. In *Abcd1*(-/-) mice, oxidative damage in the spinal cord was observed at as early as 3.5 months of age, more than 1 year before the neuropathological signs appear [95]. Deon *et al.* have reported that the total antioxidant defense was decreased in symptomatic but not in asymptomatic X-ALD patients, suggesting that asymptomatic patients might be protected against oxidative stress because of their normally functioning antioxidant defense systems [96, 97]. Taken together, the functional loss of ABCD1 could correlate with a defective antioxidant response, and oxidative damage may be linked to both the initiation and the progression of X-ALD demyelination.

In X-ALD patients, the plasmalogen level in the white matter was reported to be reduced, which might result in a susceptibility to oxidative stress [98]. Recently, it has been reported that plasmalogen functions in the protection of cells from the oxidative damage caused by VLCFA accumulation [99]. In fact, the myelin abnormalities were observed in *Abcd1*(-/-)*/Pex7*(-/-) mice had plasmalogen synthesis defects in addition to VLCFA accumulation. Taken together, it seems likely that disruption of the endogenous antioxidant system leads to reactive oxygen species (ROS) production and myelin membrane lipid oxidation, which in turn results in the inflammatory disease process.

Furthermore, Singh and colleagues have published several reports about the association of oxidative stress with the pathogenesis of X-ALD [63, 98, 100-102]. Lymphocytes from X-ALD tend to synthesize NADPH oxidase-dependent free radicals, nitric oxide and cytokines. Inflammatory cytokine expression and inducible nitrous oxide systems (NOS) were induced in *Abcd1/Abcd2*-double knockdown primary astrocytes, accompanied by an increase in C26:0 levels. Recently it was also reported that increased leukotrienes and enhanced expression of 5-lipoxygenase (5-LOX) were detected in CCALD brain, which indicated that 5-LOX might have a role in the pathology of inflammatory demyelination in CCALD [102]. VLCFA accumulation directly increases 5-LOX expression and thereby mediates the production of leukotrienes, which might be involved in astrocytic proliferation and in the cell death of oligodendrocytes. Therefore, 5-LOX might be a new target for intervention in CCALD.

In oligodendrocytes, an excess of VLCFA might trigger ROS production or reduce the resistance against oxidative stress. It is possible that a dysfunction of ABCD1 has an adverse affect on mitochondria due to the accumulation of cellular acyl-CoA, which exacerbates oxidative stress in oligodendrocyte. By selectively inactivating *PEX5* in oligodendrocytes, Kassmann *et al.* showed that the peroxisomes in oligodendrocytes are essential for the preservation of axons and the maintenance of myelin [103]. Recently, Mastroeni *et al.* have reported that viral-based delivery of insulin-like growth factor-1 and neurotrophin-3, two potent inducers of myelination and oligodendrocyte survival, respectively, halt the progression of the disease [104]. Therefore, such a functional defect of oligodendrocytes would be a target for intervention in X-ALD patients. In CCALD, however, the process of demyelination might not be cell-autonomous, because astrocytes and microglia are known to be important for the support of axonal growth and myelination. In the CNS, the expression of ABCD1 occurs mostly in astrocytes and microglia in the subcortical and cerebellar white matter. Astrocytes play a major role against oxidative stress and protect oligodendrocytes and neurons. In addition, the remarkable efficacy of HSCT in halting cerebral demyelination suggests that a dysfunction of microglial ABCD1 could contribute to demyelination, because microglial cells are derived from myelo-monocytic hematopoietic cells. Eichler *et al.* have suggested that microglial apoptosis in perilesional white matter represents an early pathogenic change in CCALD [105]. Although the exact pathway for the production of ROS in X-ALD needs to be elucidated, chemical compounds that reverse the deregulated response to oxidative stress might provide a new therapeutic approach in X-ALD. Recently, it has been reported that valproic acid has a capacity to reduce the oxidative damage in peripheral mononuclear cells from X-ALD patients. Interestingly, valproic acid did not reduce saturated VLCFA, but did reduce monounsaturated VLCFA (C26:1), suggesting that mono-unsaturated rather than saturated VLCFA is involved in the production of ROS [75]. Therefore, this drug could be an attractive candidate for X-ALD.

FUTURE APPROACHES

At present allogeneic HSCT is the only effective treatment, when performed in the early stage of cerebral demyelination, although HSCT remains associated with a high mortality risk. Furthermore, recently Cartier *et al.* have reported that lentiviral-mediated gene therapy of hematopoietic stem cells (HSC) halted the progression of X-ALD in two X-ALD boys [106]. Cerebral demyelination was arrested 14 to 16 mouths after engraftment, and neurological and cognitive functions remained stable. Compared with allogeneic HSCT, the HSC-based gene therapy, if feasible, will provide more preferable treatment for CCALD. In addition to gene therapy, pharmacological therapies are of importance in the effort to delay of onset and the progression of the disease. Considering application of various type of X-ALD, reduction of cost and risk for the therapy, and protection effect against onset of the disease, development of pharmacological therapies has many advantages. As mentioned above, pharmacological up-regulation of *ABCD2*, stimulation of peroxisomal β-oxidation, suppression of VLCFA synthesis, and reduction of oxidative damage are all promising targets for future X-ALD therapy. As target proteins for X-ALD therapy have been highlighted by recent studies, effective compounds may be identified by means of high throughput screening. At the same time, as some candidate compounds have already been found, the synthesis and evaluation of the biological activities of their derivatives should be carried forward. However, the costs in time and money to develop a new drug are formidable. The screening of well-established drug substances thus seems to be a useful means to find candidate compounds for X-ALD, since it is expected that some of these compounds will exhibit some

previously unknown efficacy against the therapeutic targets of X-ALD.

Abnormal VLCFA accumulation seems to be necessary but not sufficient for the progression of the X-ALD pathologies. It has been speculated that in addition to the defect in the *ABCD1* gene, environmental factors and/or unidentified modifier genes modulate the disease severity, since the progression and clinical symptoms of the disease in brothers with the same mutation of the *ABCD1* gene were different. Therefore, one of the factors we must keep in mind are modifier genes. However to date, while several genes have been reported to be candidates, none have been identified as actual modifier genes. The identification of triggering and/or modifying factors is important for understanding both the pathogenic mechanisms and the targeting of therapeutic compounds.

Although the precise mechanism by which the degeneration of the CNS is effected in X-ALD patients remains to be elucidated, it seems likely that an attenuation of disrupted VLCFA metabolism and a decrease in oxidative damage in the CNS have the potential to both help delay the onset of disease and prevent the progression of neurological symptoms in X-ALD. At present, pharmacological induction of ABCD2 expression is a most reasonable and feasible therapeutic strategy for X-ALD. From a practical point of view, chemical compounds with the capacity to cross the blood-brain barrier and to induce *ABCD2* gene in brain, such as halogen-free thyromimetics and histone deacetylase inhibitors, is worthwhile for a realistic application for X-ALD patients.

FOOTNOTES

This research was supported in part by a Grant-in-Aid for the Research on Measures for Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan, and for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (18590049, 18790050, and 20590054). Pacific Edit reviewed the manuscript prior to submission.

ABBREVIATIONS

ALDP = Adrenoleukodystrophy protein

ABC = ATP-binding cassette

CNS = Central nervous system

DHEA = Dehydroepiandrosterone

DHA = Docosahexaenoic acid

ELVOL = Elongation of very long chain fatty acids

ER = Endoplasmic reticulum

HSCT = Hematopoietic stem cell transplantation

IBA = Indole-3-butyric acid

5-LOX = 5-Lipoxygenase

NBD = Nucleotide binding domain

4-PBA = 4-Phenylbutyrate

PPAR = Peroxisome proliferators-activated receptor

PMP70 = 70-kDa Peroxisomal membrane protein

PUFA = Polyunsaturated fatty acid

ROS = Reactive oxygen species

SREBP = Sterol regulatory element binding protein

TDM = Transmembrane domain

TR = Thyroid hormone receptor

VLCFA = Very long chain fatty acid

X-ALD = X-Linked adrenoleukodystrophy

REFERENCES

[1] Wanders RJ, Waterham HR. Biochemistry of mammalian peroxisomes revisited. Annu Rev Biochem 2006; 75: 295-332.

[2] Visser WF, van Roermund CW, Ijlst L, Waterham HR, Wanders RJ. Metabolite transport across the peroxisomal membrane. Biochem J 2007; 401: 365-75.

[3] Mosser J, Douar AM, Sarde CO, *et al*. Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. Nature 1993; 361: 726-30.

[4] Lombard-Platet G, Savary S, Sarde CO, Mandel JL, Chimini G. A close relative of the adrenoleukodystrophy (ALD) gene codes for a peroxisomal protein with a specific expression pattern. Proc Natl Acad Sci USA 1996; 93: 1265-9.

[5] Holzinger A, Kammerer S, Berger J, Roscher AA. cDNA cloning and mRNA expression of the human adrenoleukodystrophy related protein (ALDRP), a peroxisomal ABC transporter. Biochem Biophys Res Commun 1997; 239: 261-4.

[6] Kamijo K, Taketani S, Yokota S, Osumi T, Hashimoto T. The 70-kDa peroxisomal membrane protein is a member of the Mdr (P-glycoprotein)-related ATP-binding protein superfamily. J Biol Chem 1990; 265: 4534-40.

[7] Kamijo K, Kamijo T, Ueno I, Osumi T, Hashimoto T. Nucleotide sequence of the human 70 kDa peroxisomal membrane protein: a member of ATP-binding cassette transporters. Biochim Biophys Acta 1992; 1129: 323-7.

[8] Gartner J, Moser H, Valle D. Mutations in the 70K peroxisomal membrane protein gene in Zellweger syndrome. Nat Genet 1992; 1: 16-23.

[9] Cartier N, Lopez J, Moullier P, *et al*. Retroviral-mediated gene transfer corrects very-long-chain fatty acid metabolism in adrenoleukodystrophy fibroblasts. Proc Natl Acad Sci USA 1995; 92: 1674-8.

[10] Braiterman LT, Zheng S, Watkins PA, *et al*. Suppression of peroxisomal membrane protein defects by peroxisomal ATP binding cassette (ABC) proteins. Hum Mol Genet 1998; 7: 239-47.

[11] Unterrainer G, Molzer B, Forss-Petter S, Berger J. Co-expression of mutated and normal adrenoleukodystrophy protein reduces protein function: implications for gene therapy of X-linked adrenoleukodystrophy. Hum Mol Genet 2000; 9: 2609-16.

[12] Netik A, Forss-Petter S, Holzinger A, Molzer B, Unterrainer G, Berger J. Adrenoleukodystrophy-related protein can compensate functionally for adrenoleukodystrophy protein deficiency (X-ALD): implications for therapy. Hum Mol Genet 1999; 8: 907-13.

[13] Imanaka T, Aihara K, Takano T, *et al*. Characterization of the 70-kDa peroxisomal membrane protein, an ATP binding cassette transporter. J Biol Chem 1999; 274: 11968-76.

[14] Shani N, Jimenez-Sanchez G, Steel G, Dean M, Valle D. Identification of a fourth half ABC transporter in the human peroxisomal membrane. Hum Mol Genet 1997; 6: 1925-31.

[15] Kashiwayama Y, Seki M, Yasui A, *et al*. 70-kDa peroxisomal membrane protein related protein (P70R/ABCD4) localizes to endoplasmic reticulum not peroxisomes, and NH_2-terminal hydrophobic property determines the subcellular localization of ABC subfamily D proteins. Exp Cell Res 2009; 315: 190-205.

[16] Aubourg P, Adamsbaum C, Lavallard-Rousseau MC, *et al*. A two-year trial of oleic and erucic acids ("Lorenzo's oil") as treatment for adrenomyeloneuropathy. N Engl J Med 1993; 329: 745-52.

[17] Asano J, Suzuki Y, Yajima S, *et al*. Effects of erucic acid therapy on Japanese patients with X-linked adrenoleukodystrophy. Brain Dev 1994; 16: 454-8.

[18] Peters C, Charnas LR, Tan Y, *et al*. Cerebral X-linked adrenoleukodystrophy: the international hematopoietic cell transplantation experience from 1982 to 1999. Blood 2004; 104: 881-8.

[19] Contreras M, Sengupta TK, Sheikh F, Aubourg P, Singh I. Topology of ATP-binding domain of adrenoleukodystrophy gene product in peroxisomes. Arch Biochem Biophys 1996; 334: 369-79.

[20] Roerig P, Mayerhofer P, Holzinger A, Gartner J. Characterization and functional analysis of the nucleotide binding fold in human peroxisomal ATP binding cassette transporters. FEBS Lett 2001; 492: 66-72.

[21] Tanaka AR, Tanabe K, Morita M, *et al*. ATP binding/hydrolysis by and phosphorylation of peroxisomal ATP-binding cassette proteins PMP70 (ABCD3) and adrenoleukodystrophy protein (ABCD1). J Biol Chem 2002; 277: 40142-7.

[22] Guimarães CP, Sa-Miranda C, Azevedo JE. Probing substrate-induced conformational alterations in adrenoleukodystrophy protein by proteolysis. J Hum Genet 2005; 50: 99-105.

[23] Liu LX, Janvier K, Berteaux-Lecellier V, Cartier N, Benarous R, Aubourg P. Homo- and heterodimerization of peroxisomal ATP-binding cassette half-transporters. J Biol Chem 1999; 274: 32738-43.

[24] Guimaraes CP, Domingues P, Aubourg P, *et al*. Mouse liver PMP70 and ALDP: homomeric interactions prevail *in vivo*. Biochim Biophys Acta 2004; 1689: 235-43.

[25] Hillebrand M, Verrier SE, Ohlenbusch A, *et al*. Live cell FRET microscopy: homo- and heterodimerization of two human peroxisomal ABC transporters, the adrenoleukodystrophy protein (ALDP, ABCD1) and PMP70 (ABCD3). J Biol Chem 2007; 282: 26997-7005.

[26] Singh I, Moser AE, Goldfischer S, Moser HW. Lignoceric acid is oxidized in the peroxisome: implications for the Zellweger cerebro-hepato-renal syndrome and adrenoleukodystrophy. Proc Natl Acad Sci USA 1984; 81: 4203-7.

[27] Kobayashi T, Shinnoh N, Kondo A, Yamada T. Adrenoleukodystrophy protein-deficient mice represent abnormality of very long chain fatty acid metabolism. Biochem Biophys Res Commun 1997; 232: 631-6.

[28] Forss-Petter S, Werner H, Berger J, *et al*. Targeted inactivation of the X-linked adrenoleukodystrophy gene in mice. J Neurosci Res 1997; 50: 829-43.

[29] Lu JF, Lawler AM, Watkins PA, *et al*. A mouse model for X-linked adrenoleukodystrophy. Proc Natl Acad Sci USA 1997; 94: 9366-71.

[30] Braiterman LT, Watkins PA, Moser AB, Smith KD. Peroxisomal very long chain fatty acid β-oxidation activity is determined by the level of adrenodeukodystrophy protein (ALDP) expression. Mol Genet Metab 1999; 66: 91-9.

[31] van Roermund CW, Visser WF, Ijlst L, *et al*. The human peroxisomal ABC half transporter ALDP functions as a homodimer and accepts acyl-CoA esters. FASEB J 2008; 22: 4201-8.

[32] Shani N, Watkins PA, Valle D. PXA1, a possible Saccharomyces cerevisiae ortholog of the human adrenoleukodystrophy gene. Proc Natl Acad Sci USA 1995; 92: 6012-6.

[33] Swartzman EE, Viswanathan MN, Thorner J. The PAL1 gene product is a peroxisomal ATP-binding cassette transporter in the yeast Saccharomyces cerevisiae. J Cell Biol 1996; 132: 549-63.

[34] Hettema EH, van Roermund CW, Distel B, *et al*. The ABC transporter proteins Pat1 and Pat2 are required for import of long-chain fatty acids into peroxisomes of *Saccharomyces cerevisiae*. EMBO J 1996; 15: 3813-22.

[35] Verleur N, Hettema EH, van Roermund CW, Tabak HF, Wanders RJ. Transport of activated fatty acids by the peroxisomal ATP-binding-cassette transporter Pxa2 in a semi-intact yeast cell system. Eur J Biochem 1997; 249: 657-61.

[36] Zolman BK, Silva ID, Bartel B. The Arabidopsis pxa1 mutant is defective in an ATP-binding cassette transporter-like protein required for peroxisomal fatty acid beta-oxidation. Plant Physiol 2001; 127: 1266-78.

[37] Footitt S, Slocombe SP, Larner V, *et al*. Control of germination and lipid mobilization by COMATOSE, the Arabidopsis homologue of human ALDP. EMBO J 2002; 21: 2912-22.

[38] Kunz HH, Scharnewski M, Feussner K, *et al*. The ABC transporter PXA1 and peroxisomal β-oxidation are vital for metabolism in mature leaves of Arabidopsis during extended darkness. Plant Cell 2009; 21: 2733-49.

[39] Migeon BR, Moser HW, Moser AB, Axelman J, Sillence D, Norum RA. Adrenoleukodystrophy: evidence for X linkage, inactivation, and selection favoring the mutant allele in heterozygous cells. Proc Natl Acad Sci USA 1981; 78: 5066-70.

[40] Kemp S, Pujol A, Waterham HR, *et al*. ABCD1 mutations and the X-linked adrenoleukodystrophy mutation database: role in diagnosis and clinical correlations. Hum Mutat 2001; 18: 499-515.

[41] Takahashi N, Morita M, Maeda T, *et al*. Adrenoleukodystrophy: subcellular localization and degradation of adrenoleukodystrophy protein (ALDP/ABCD1) with naturally occurring missense mutations. J Neurochem 2007; 101: 1632-43.

[42] Moser HW, Mahmood A, Raymond GV. X-linked adrenoleukodystrophy. Nat Clin Pract Neurol 2007; 3: 140-51.

[43] Takemoto Y, Suzuki Y, Tamakoshi A, *et al*. Epidemiology of X-linked adrenoleukodystrophy in Japan. J Hum Genet 2002; 47: 590-3.

[44] Smith KD, Kemp S, Braiterman LT, *et al*. X-linked adrenoleukodystrophy: genes, mutations, and phenotypes. Neurochem Res 1999; 24: 521-35.

[45] Suzuki Y, Takemoto Y, Shimozawa N, *et al*. Natural history of X-linked adrenoleukodystrophy in Japan. Brain Dev 2005; 27: 353-7.

[46] Ohno T, Tsuchida H, Fukuhara N, *et al*. Adrenoleukodystrophy: a clinical variant presenting as olivopontocerebellar atrophy. J Neurol 1984; 231: 167-9.

[47] Ofman R, Dijkstra IM, van Roermund CW, *et al*. The role of ELOVL1 in very long-chain fatty acid homeostasis and X-linked adrenoleukodystrophy. EMBO Mol Med 2010; 2: 90-7.

[48] Moser HW. Adrenoleukodystrophy: phenotype, genetics, pathogenesis and therapy. Brain 1997; 120: 1485-508.

[49] Whitcomb RW, Linehan WM, Knazek RA. Effects of long-chain, saturated fatty acids on membrane microviscosity and adrenocorticotropin responsiveness of human adrenocortical cells *in vitro*. J Clin Invest 1988; 81: 185-8.

[50] Hein S, Schonfeld P, Kahlert S, Reiser G. Toxic effects of X-linked adrenoleukodystrophy-associated, very long chain fatty acids on glial cells and neurons from rat hippocampus in culture. Hum Mol Genet 2008; 17: 1750-61.

[51] Martinez M, Vazquez E. MRI evidence that docosahexaenoic acid ethyl ester improves myelination in generalized peroxisomal disorders. Neurology 1998; 51: 26-32.

[52] Ito M, Blumberg BM, Mock DJ, *et al*. Potential environmental and host participants in the early white matter lesion of adreno-leukodystrophy: morphologic evidence for CD8 cytotoxic T cells, cytolysis of oligodendrocytes, and CD1-mediated lipid antigen presentation. J Neuropathol Exp Neurol 2001; 60: 1004-19.

[53] Gilg AG, Singh AK, Singh I. Inducible nitric oxide synthase in the central nervous system of patients with X-adrenoleukodystrophy. J Neuropathol Exp Neurol 2000; 59: 1063-9.

[54] Powers JM, Pei Z, Heinzer AK, *et al*. Adreno-leukodystrophy: oxidative stress of mice and men. J Neuropathol Exp Neurol 2005; 64: 1067-79.

[55] Hubbard WC, Moser AB, Liu AC, *et al*. Newborn screening for X-linked adrenoleukodystrophy (X-ALD): validation of a combined liquid chromatography-tandem mass spectrometric (LC-MS/MS) method. Mol Genet Metab 2009; 97: 212-20.

[56] Moser HW, Moser AB, Hollandsworth K, Brereton NH, Raymond GV. "Lorenzo's oil" therapy for X-linked adrenoleukodystrophy: rationale and current assessment of efficacy. J Mol Neurosci 2007; 33: 105-13.

[57] Deon M, Garcia MP, Sitta A, *et al*. Hexacosanoic and docosanoic acids plasma levels in patients with cerebral childhood and asymptomatic X-linked adrenoleukodystrophy: Lorenzo's oil effect. Metab Brain Dis 2008; 23: 43-9.

[58] Semmler A, Bao X, Cao G, *et al*. Genetic variants of methionine metabolism and X-ALD phenotype generation: results of a new study sample. J Neurol 2009; 256: 1277-80.

[59] Golovko MY, Murphy EJ. Uptake and metabolism of plasma-derived erucic acid by rat brain. J Lipid Res 2006; 47: 1289-97.

[60] Pai GS, Khan M, Barbosa E, *et al*. Lovastatin therapy for X-linked adrenoleukodystrophy: clinical and biochemical observations on 12 patients. Mol Genet Metab 2000; 69: 312-22.

[61] Yamada T, Shinnoh N, Taniwaki T, *et al*. Lovastatin does not correct the accumulation of very long-chain fatty acids in tissues of adrenoleukodystrophy protein-deficient mice. J Inherit Metab Dis 2000; 23: 607-14.

[62] Cartier N, Guidoux S, Rocchiccioli F, Aubourg P. Simvastatin does not normalize very long chain fatty acids in adrenoleukodystrophy mice. FEBS Lett 2000; 478: 205-8.

[63] Uto T, Contreras MA, Gilg AG, Singh I. Oxidative imbalance in nonstimulated X-adrenoleukodystrophy-derived lymphoblasts. Dev Neurosci 2008; 30: 410-8.

[64] Engelen M, Ofman R, Dijkgraaf MG, *et al*. Lovastatin in X-linked adrenoleukodystrophy. N Engl J Med 2010; 362: 276-7.

[65] Pujol A, Ferrer I, Camps C, *et al*. Functional overlap between ABCD1 (ALD) and ABCD2 (ALDR) transporters: a therapeutic target for X-adrenoleukodystrophy. Hum Mol Genet 2004; 13: 2997-3006.

[66] Maier EM, Mayerhofer PU, Asheuer M, *et al*. X-linked adreno-leukodystrophy phenotype is independent of ABCD2 genotype. Biochem Biophys Res Commun 2008; 377: 176-80.

[67] Weinhofer I, Kunze M, Rampler H, *et al*. Distinct modulatory roles for thyroid hormone receptors TRα and TRβ in SREBP1-activated ABCD2 expression. Eur J Cell Biol 2008; 87: 933-45.

[68] Weinhofer I, Forss-Petter S, Zigman M, Berger J. Cholesterol regulates ABCD2 expression: implications for the therapy of X-linked adrenoleukodystrophy. Hum Mol Genet 2002; 11: 2701-8.

[69] Weinhofer I, Kunze M, Rampler H, Bookout AL, Forss-Petter S, Berger J. Liver X receptor α interferes with SREBP1c-mediated Abcd2 expression. Novel cross-talk in gene regulation. J Biol Chem 2005; 280: 41243-51.

[70] Genin EC, Gondcaille C, Trompier D, Savary S. Induction of the adrenoleukodystrophy-related gene (ABCD2) by thyromimetics. J Steroid Biochem Mol Biol 2009; 116: 37-43.

[71] Gueugnon F, Gondcaille C, Leclercq S, *et al*. Dehydroepiandro-sterone up-regulates the Adrenoleukodystrophy-related gene (ABCD2) independently of PPARα in rodents. Biochimie 2007; 89: 1312-21.

[72] Hahnen E, Hauke J, Trankle C, Eyupoglu IY, Wirth B, Blumcke I. Histone deacetylase inhibitors: possible implications for neurodegenerative disorders. Expert Opin Investig Drugs 2008; 17: 169-84.

[73] McGuinness MC, Zhang HP, Smith KD. Evaluation of pharmacological induction of fatty acid β-oxidation in X-linked adrenoleukodystrophy. Mol Genet Metab 2001; 74: 256-63.

[74] Kemp S, Wanders RJ. X-linked adrenoleukodystrophy: very long-chain fatty acid metabolism, ABC half-transporters and the complicated route to treatment. Mol Genet Metab 2007; 90: 268-76.

[75] Fourcade S, Ruiz M, Guilera C, *et al*. Valproic acid induces antioxidant effects in X-linked adrenoleukodystrophy. Hum Mol Genet 2010; 19: 2005-14.

[76] Engelen M, Ofman R, Mooijer PA, Poll-The BT, Wanders RJ, Kemp S. Cholesterol-deprivation increases mono-unsaturated very long-chain fatty acids in skin fibroblasts from patients with X-linked adrenoleukodystrophy. Biochim Biophys Acta 2008; 1781: 105-11.

[77] Leclercq S, Skrzypski J, Courvoisier A, *et al*. Effect of dietary polyunsaturated fatty acids on the expression of peroxisomal ABC transporters. Biochimie 2008; 90: 1602-7.

[78] Fourcade S, Ruiz M, Camps C, *et al*. A key role for the peroxisomal ABCD2 transporter in fatty acid homeostasis. Am J Physiol Endocrinol Metab 2009; 296: E211-21.

[79] Lu JF, Barron-Casella E, Deering R, *et al*. The role of peroxisomal ABC transporters in the mouse adrenal gland: the loss of Abcd2 (ALDR), Not Abcd1 (ALD), causes oxidative damage. Lab Invest 2007; 87: 261-72.

[80] Di Benedetto R, Denti MA, Salvati S, Attorri L, Di Biase A. PMP70 knock-down generates oxidative stress and pro-inflammatory cytokine production in C6 glial cells. Neurochem Int 2009; 54: 37-42.

[81] Asheuer M, Bieche I, Laurendeau I, *et al*. Decreased expression of ABCD4 and BG1 genes early in the pathogenesis of X-linked adrenoleukodystrophy. Hum Mol Genet 2005; 14: 1293-303.

[82] Liu XL, Done SC, Yan K, Kilpelainen P, Pikkarainen T, Tryggvason K. Defective trafficking of nephrin missense mutants rescued by a chemical chaperone. J Am Soc Nephrol 2004; 15: 1731-8.

[83] Lim M, McKenzie K, Floyd AD, Kwon E, Zeitlin PL. Modulation of deltaF508 cystic fibrosis transmembrane regulator trafficking and function with 4-phenylbutyrate and flavonoids. Am J Respir Cell Mol Biol 2004; 31: 351-7.

[84] Pillai BK, Jasuja R, Simard JR, Hamilton JA. Fast diffusion of very long chain saturated fatty acids across a bilayer membrane and their rapid extraction by cyclodextrins: implications for adrenoleuko-dystrophy. J Biol Chem 2009; 284: 33296-304.

[85] Morita M, Takahashi I, Kanai M, *et al*. Baicalein 5,6,7-trimethyl ether, a flavonoid derivative, stimulates fatty acid β-oxidation in skin fibroblasts of X-linked adrenoleukodystrophy. FEBS Lett 2005; 579: 409-14.

[86] Morita M, Kanai M, Mizuno S, *et al*. Baicalein 5,6,7-trimethyl ether activates peroxisomal but not mitochondrial fatty acid β-oxidation. J Inherit Metab Dis 2008; 31: 442-9.

[87] Roessmann U, Hori A. Agyria (lissencephaly) with anomalous pyramidal crossing. Case report and review of literature. J Neurol Sci 1985; 69: 357-64.

[88] Koike R, Tsuji S, Ohno T, Suzuki Y, Orii T, Miyatake T. Physiological significance of fatty acid elongation system in adrenoleukodystrophy. J Neurol Sci 1991; 103: 188-94.

[89] Rizzo WB, Watkins PA, Phillips MW, Cranin D, Campbell B, Avigan J. Adrenoleukodystrophy: oleic acid lowers fibroblast saturated C22-26 fatty acids. Neurology 1986; 36: 357-61.

[90] Jakobsson A, Westerberg R, Jacobsson A. Fatty acid elongases in mammals: their regulation and roles in metabolism. Prog Lipid Res 2006; 45: 237-49.

[91] Kitazawa H, Miyamoto Y, Shimamura K, Nagumo A, Tokita S. Development of a high-density assay for long-chain fatty acyl-CoA elongases. Lipids 2009; 44: 765-73.

[92] Tamura K, Makino A, Hullin-Matsuda F, *et al*. Novel lipogenic enzyme ELOVL7 is involved in prostate cancer growth through saturated long-chain fatty acid metabolism. Cancer Res 2009; 69: 8133-40.

[93] Kemp S, Valianpour F, Denis S, *et al*. Elongation of very long-chain fatty acids is enhanced in X-linked adrenoleukodystrophy. Mol Genet Metab 2005; 84: 144-51.

[94] Kumadaki S, Matsuzaka T, Kato T, *et al*. Mouse Elovl-6 promoter is an SREBP target. Biochem Biophys Res Commun 2008; 368: 261-6.

[95] Fourcade S, Lopez-Erauskin J, Galino J, *et al*. Early oxidative damage underlying neurodegeneration in X-adrenoleukodystrophy. Hum Mol Genet 2008; 17: 1762-73.

[96] Deon M, Sitta A, Barschak AG, *et al*. Oxidative stress is induced in female carriers of X-linked adrenoleukodystrophy. J Neurol Sci 2008; 266: 79-83.

[97] Deon M, Sitta A, Barschak AG, *et al*. Induction of lipid peroxidation and decrease of antioxidant defenses in symptomatic and asymptomatic patients with X-linked adrenoleukodystrophy. Int J Dev Neurosci 2007; 25: 441-4.

[98] Khan M, Singh J, Singh I. Plasmalogen deficiency in cerebral adrenoleukodystrophy and its modulation by lovastatin. J Neurochem 2008; 106: 1766-79.

[99] Brites P, Mooyer PA, El Mrabet L, Waterham HR, Wanders RJ. Plasmalogens participate in very-long-chain fatty acid-induced pathology. Brain 2009; 132: 482-92.

[100] Singh I, Singh AK, Contreras MA. Peroxisomal dysfunction in inflammatory childhood white matter disorders: an unexpected contributor to neuropathology. J Child Neurol 2009; 24: 1147-57.

[101] Singh J, Khan M, Singh I. Silencing of Abcd1 and Abcd2 genes sensitizes astrocytes for inflammation: implication for X-adrenoleukodystrophy. J Lipid Res 2009; 50: 135-47.

[102] Khan M, Singh J, Gilg AG, Uto T, Singh I. Very long chain fatty acid accumulation causes lipotoxic response via 5-lipoxygenase in cerebral adrenoleukodystrophy. J Lipid Res 2010; 51: 1685-95.

[103] Kassmann CM, Lappe-Siefke C, Baes M, *et al*. Axonal loss and neuroinflammation caused by peroxisome-deficient oligodendrocytes. Nat Genet 2007; 39: 969-76.

[104] Mastroeni R, Bensadoun JC, Charvin D, Aebischer P, Pujol A, Raoul C. Insulin-like growth factor-1 and neurotrophin-3 gene therapy prevents motor decline in an X-linked adrenoleukodystrophy mouse model. Ann Neurol 2009; 66: 117-22.

[105] Eichler FS, Ren JQ, Cossoy M, *et al*. Is microglial apoptosis an early pathogenic change in cerebral X-linked adrenoleukodystrophy? Ann Neurol 2008; 63: 729-42.

[106] Cartier N, Hacein-Bey-Abina S, Bartholomae CC, *et al*. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. Science 2009; 326: 818-23.

Received: May 16, 2010 Revised: August 17, 2010 Accepted: August 17, 2010

PMID: 21039332