5 ABC Drug Transporters and Their Impact on Drug Disposition/Drug Sensitivity and Resistance

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5.1 SUMMARY

Of the 49 members of the ATP-binding cassette (ABC) family of proteins, the majority harness the energy of ATP hydrolysis to facilitate a unidirectional efflux of substrates from the cytoplasm and inner leaflet of the lipid bilayer to the extracellular space. Each of these proteins share highly conserved amino acid sequences that permit their classification into the ABC superfamily. These motifs lie within the nucleotide binding domains (NBDs) and include the Walker A, Walker B, and Signature C motifs. Importantly, their structures differ substantially in the substrate binding regions, ensuring the transport of a wide range of substrates. A large number of small-molecule therapeutics have been identified as substrates of at least one ABC transport pump. P-glycoprotein (P-gp), which is encoded by the *ABCB1* gene; multidrug-resistance-associated protein 1 (MRP1), which is encoded by the *ABCC1* gene; and breast cancer resistance protein, which is encoded by the *ABCG2* gene, in particular, are known to confer a multidrug resistance (MDR) phenotype. Therapeutics vulnerable to efflux

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by this family of proteins include the anthracycline antibiotics and tyrosine kinase inhibitors used in the treatment of cancer, antivirals used to treat HIV, antihistamines used in the treatment of allergies, and immunosuppressants used to prevent graft rejection. Despite their well-established role in the onset of drug resistance, reversal of the MDR phenomenon by targeting the proteins through molecular mechanisms such as small interfering RNA (siRNA) or small-molecule inhibitors such as verapamil, or the more specific second- and third-generation inhibitors such as dexverapamil and tariquidar, respectively, have yielded limited results. To date, the ABC transporters are not targeted in adjuvant therapy and MDR continues to hamper many chemotherapy regimes. Further confounding the role of these proteins in drug response is genetic variation, particularly, single nucleotide polymorphisms (SNPs). This variation, which is often evident in populations of distinct evolutionary history, may manifest itself either through differences in drug efficacy between populations or through the onset of adverse effects. While the effect of such genetic variation is often subtle, loss-of-function mutations in the ABC genes are also common and may lead to severe diseases. Examples include cystic fibrosis (CF) and Tangier disease, which result from mutations in *ABCC7* and *ABCA1*, respectively. Each member of the ABC superfamily contributes to drug resistance and/or drug response in a unique manner that is dependent on the protein location, the specific drug, and the genetic history of the individual. Within this chapter, the role of the ABC transport proteins on drug efficacy and resistance is explored. Efforts to disrupt ABC-mediated drug resistance are discussed, and the influence of polymorphisms and mutations on protein function is also assessed.

5.2 INTRODUCTION TO THE ABC FAMILY OF TRANSPORT PROTEINS

5.2.1 Classification of the ABC Family

The ABC superfamily is the largest family of membrane-bound proteins in biology. To date, 49 members have been identified, and although some have functions other than substrate transport across lipid bilayers, each possess key sequence motifs and ATP-binding domain organization that permits their classification into one of seven subclasses, ABCA–ABCG [1,2]. While many of the genes that encode the ABC proteins are scattered throughout the genome, several reside in close proximity to each other, most often encoding members of the same subclass. Furthermore, while some of the ABC transport proteins are expressed ubiquitously throughout the body, others remain localized to specific tissue types where they transport physiological substrates that are required by that particular tissue or organ. A complete summary of ABC localization and function is presented in Table 5.1.

5.2.2 Protein Structure and Function

In eukaryotic cells, the majority of the ABC transport proteins are responsible for a unidirectional, active transfer of substrates across lipid bilayers, from the cytoplasm to the extracellular space. In some instances, where ABC proteins are localized to the lipid bilayer membranes of intracellular vesicles such as lysosomes or endosomes, substrates are trafficked from the cytoplasm into the vesicular compartments for further processing or efflux into the extracellular space [12,13]. Members of the ABCE and

TABLE 5.1 Location of Genes Encoding Members of the ABC Superfamily of Proteins, their Cellular/Tissue Localization and their Physiological Substrates

Gene Name	Gene Location	Cellular/Tissue Localization	Physiological Substrate/Function	References
ABCA1	9q31.1	Brain and liver	Lipids (HDL and cholesterol)	1–4
ABCA2	9p34	Brain, monocytes	Steroid derivatives, lipids	
ABCA3	16p13.3	Lung (alveolar type II cells)	Pulmonary surfactant	
ABCA4	1p22.1-p21	Retina	Retinylidene phospholipid complexes	
ABCA5	17q24	Intracellular localization; skeletal muscle, kidney, liver, placenta	Unknown; possibly lysosomal substrates	
ABCA6	17q24	Ubiquitous	Unknown; possibly macrophage lipids	
ABCA7	19p13.3	Myelolymphatic tissues, keratinocytes	Phospholipids, including phosphatidylcholine	
ABCA8	17q24	Heart, skeletal muscle, liver	Unknown	
ABCA9	17q24	Ubiquitous	Unknown	
ABCA10	17q24	Ubiquitous, particularly in the intestinal tract	Unknown; possibly lipid transport	
ABCA12	2q34	Keratinocytes, stomach	Lipids	
ABCA13	7p11-q11	Trachea, testis, bone marrow	Unknown	
ABCB1	7q21	Intestine, liver, kidney, placenta, Blood brain barrier (BBB)	Multidrug resistance	1–3, 5,6
ABCB2	6p21	All cells	Peptide transport	
ABCB3	6p21	All cells	Peptide transport	
ABCB4	7q21.1	Liver	Unknown	
ABCB5	7p14	Ubiquitous	Unknown	1–3, 5
ABCB6	2q36	Mitochondria	Iron transport	
ABCB7	Xq12-q13	Mitochondria	Fe/S cluster transport	
ABCB8	7q36	Mitochondria	Unknown	
ABCB9	12q24	Heart, brain	Unknown	
ABCB10	1q24	Mitochondria	Unknown	
ABCB11	2q24	Liver	Bile salts	2,7,8
ABCC1	16p13.1	Ubiquitous	Organic anion efflux	1–3, 5

(*continued overleaf*)

TABLE 5.1 (*Continued*)

Gene Name	Gene Location	Cellular/Tissue Localization	Physiological Substrate/Function	References
ABCC2	10q24	Liver, kidney, intestine	Organic anion efflux	
ABCC3	17q21.3	Pancreas, kidney, intestine, liver, adrenal gland	Organic anion efflux	
ABCC4	13q32	Prostate, testis, ovary, intestine, pancreas, lung	Nucleoside transport	
ABCC5	3q27	Ubiquitous	Nucleoside transport	
ABCC6	16p13.1	Liver, kidney	Unknown	
ABCC7	7q31.2	Exocrine tissue	Chloride ion channel	
ABCC8	11p15.1	Pancreas	Sulfonylurea receptor	
ABCC9	12p12.1	Heart, muscle	Unknown	
ABCC10	6p21	Liver, heart, kidney	Unknown	
ABCC11	16q11-q12	Ubiquitous	Unknown	
ABCC12	16q11-q12	Ubiquitous	Unknown	
ABCD1	Xq28	Peroxisomes; ubiquitous	Acyl CoA, VLCFAs	9
ABCD2	12q11-q12	Peroxisomes; ubiquitous	VLCFA CoAs	
ABCD3	1p22-p21	Peroxisomes; ubiquitous	LCFA CoAs	
ABCD4	14q24.3	Peroxisomes	Unknown	
ABCE1	4q31	Ovary, testes, spleen	Oligoadenylate binding protein	2,10
ABCF1	6p21.33	Ubiquitous	Not Applicable (NA)	2
ABCF2	7q36	Ubiquitous	NA	
ABCF3	3q25	Ubiquitous	NA	
ABCG1	21q22.3	Adipocytes, macrophages	Cholesterol and Phospholipids	2,11
ABCG2	4q22	Placenta, intestine, breast, liver	Toxin efflux, drug resistance	
ABCG4	11q23	Adipocytes, macrophages	Cholesterol and phospholipids	
ABCG5	2p21	Liver hepatocytes, enterocytes in small intestine and colon	Lipophilic cholesterol	
ABCG8	2p21	Liver hepatocytes, knterocytes in small intestine and colon	Lipophilic cholesterol	

Abbreviations: VLCFA, very-long-chain fatty acid; LCFA, long-chain fatty acid.
Source: Adapted from Ref. 2.

ABCF subclasses do not function as membrane-bound transporters but are instead involved in ribosome biogenesis and translation initiation and elongation [10,14]. As these two subclasses do not influence the pharmacokinetics of drugs, they are not discussed further in this chapter.

Functional ABC transport proteins possess a minimum of two transmembrane domains (TMDs) with each TMD containing four to eight transmembrane (TM) helices. These not only permit a passageway for substrate transport but also form a cavity for drug binding known as the ligand binding domain (LBD) [3]. Attached to the TMDs are cytosolic NBDs that facilitate the binding and hydrolysis of ATP. By harnessing the energy of ATP hydrolysis, active transport of substrates across lipid bilayers, and often against chemical gradients, may occur [5]. Although the specific mechanism of substrate transport has not been completely defined, it is believed that a conformational change in the arrangement of the TMDs occurs following NBD dimerization. This subsequently alters substrate binding affinity and opens up the LBD to the extracellular space [15].

The NBDs, unlike the TMDs, are highly homologous across the ABC superfamily and feature a number of sequence motifs essential for protein function. The Walker A motif and the A-loop, which is a single tyrosine residue ~25 amino acids upstream of Walker A [16], have both been shown to be essential for ATP binding and hydrolysis [5,17,18], while other sequences including Walker B, the D-loop, H-loop, Q-loop, and the Signature C motif (LSGGQ), which is unique to the ABC transport proteins [19], are believed to bind diatonic metals such as Mg^{2+} and contribute to the binding and hydrolysis of ATP via their arrangement during NBD dimerization [16,20]. ATP initially binds to only a single NBD before dimerization of the two NBD occurs [21]. During dimerization, ATP is sandwiched between the two NBDs, in a binding cavity composed of the Signature C motif and D-loop from one NBD and the Walker A, Walker B, A-loop, H-loop, and Q-loop from the other NBD [22].

A number of ABC transport proteins are known as *half-transport proteins* and comprise only a single TMD and NBD each (Fig. 5.1) These include ABCB2, ABCB3, ABCB6, ABCB7, ABCB8, ABCB9, and ABCB10, as well as the four members of the ABCD subclass and the five members of the ABCG subclass [3]. In these cases, transport function is only acquired after dimerization between two or three half-transport protein members of the same subclass. In some instances, a homodimer may form, such as with ABCD1 [23] and BCRP (breast cancer resistance protein) [24]. In other cases, however, heterodimer complexes will be formed before integration into the lipid bilayers. This protein interaction was shown to exist between ABCG5 and ABCG8 [25], and it is well established that ABCB2 forms a functional heterodimer with ABCB3 [3].

With the exception of those in the ABCE and ABCF subclasses, the remaining ABC proteins are known as *full transporters*, each possessing at least two TMDs and two NBDs (Fig. 5.1). Each member of the ABCA subclass possesses this general structure as does the well-established ABC transporter P-gp, which was originally discovered in 1976 in MDR Chinese hamster ovary (CHO) cells [26]. The crystallization of mouse P-gp, which features 87% sequence homology to human P-gp, highlighted many of the structural features that give rise to protein function. These included substrate "entry portals" that are open to the inner leaflet of the lipid bilayer and ligand binding cavity of ~6000 $Å^3$ that houses 73 residues capable of substrate binding [27]. A smaller group of "full transporters" are all classified into the ABCC subclass feature an additional N-terminal TMD and linker region (Fig. 5.1). These include ABCC1, ABCC2, ABCC3,

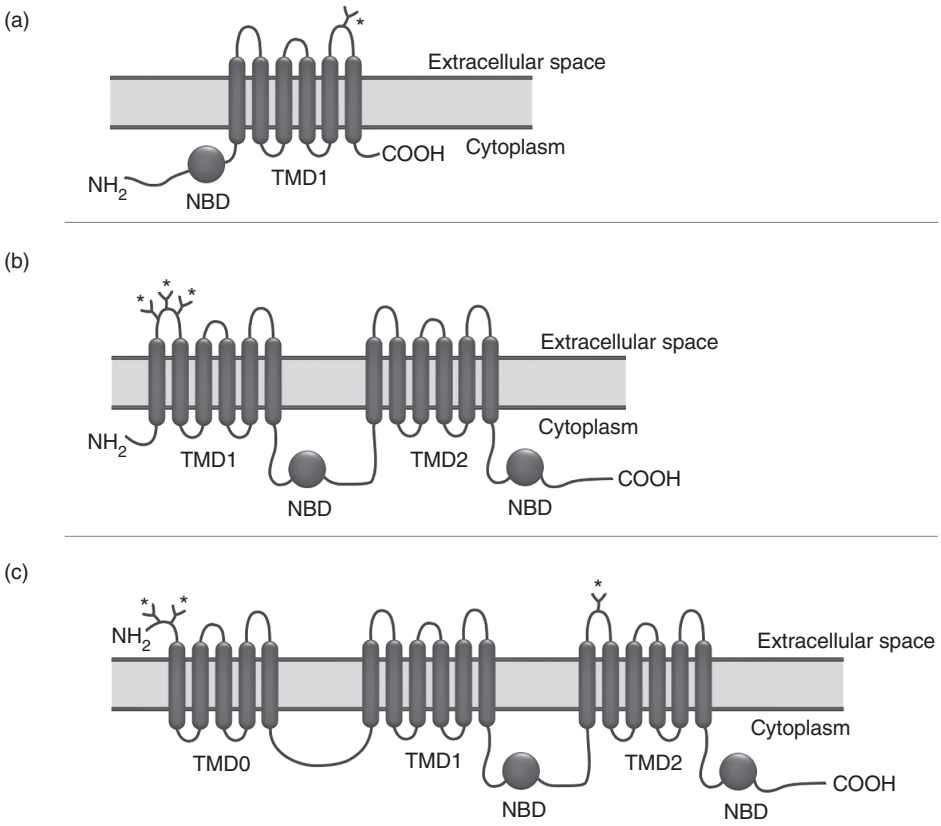

Figure 5.1 Schematic diagram showing the topology of the three major ABC transport proteins. (a) ABCG2 (BCRP) is a half transporter, comprising only a single transmembrane domain made up of six transmembrane helices and a single cytoplasmic nucleotide binding domain. (b) ABCB1 (P-glycoprotein) is a full transporter with two transmembrane domains and two nucleotide binding domains. (c) ABCC1 (MRP1) is also a full transporter but with an additional transmembrane domain (TM0) comprising five transmembrane helices. Each of these proteins contains glycosylation sites that are indicated by "*." *Source*: Adapted from Ref. 5. (See color insert.)

ABCC6, ABCC8, ABCC9, and ABCC10. MRP1 is one of the most well-established ABC transport proteins possessing this structural property. Sharing less than 20% amino acid identity with P-gp [28], MRP1 features not only an additional TMD, which comprises five TM segments [29], but also a 13-residue amino acid sequence deletion between the Walker A and Walker B sequence motifs in the NBD1 [28]. The importance of this deletion was made evident when it was shown that insertion of the 13 residues from P-gp renders MRP1 inactive [30]. Although the additional cytoplasmic linker region (L_0) was shown to be vital for substrate transport, loss of TMD_0 does not greatly affect substrate transport [31]. Two other "full transporters" possessing an additional TMD are the sulfonylurea receptors ABCC8 and ABCC9. These proteins function only in complex with their K^+ channel counterparts despite possessing three TMDs. Each protein remains in the cytoplasm until being activated by ATP/ADP, at which point hetero-octameric complexes are formed with the potassium channels Kir6.1 and Kir6.2,

respectively. It is in this position that ABCC8 and ABCC9 regulate the permeability of these potassium channels [32].

5.3 TRANSPORT OF SMALL MOLECULES BY ABC PROTEINS

5.3.1 Specificity of Substrate Binding

Most pharmacological substrates are transported by more than one ABC protein. As highlighted in Table 5.2, many anticancer drugs are vulnerable to efflux via four well-researched ABC drug transporters; P-gp, MRP1, MRP2, and BCRP. For example, the well-established topoisomerase poisons, etoposide and teniposide, are substrates of these three ABC drug transporters, while the taxanes, paclitaxel and docetaxel, are not known to be substrates of either MRP1 or BCRP, yet are transported by P-gp [5].

Generally, P-gp will transport neutral or positively charged hydrophobic molecules, whereas MRP1 is predominately an anionic transporter [51]. Binding and efflux of substrates is, however, selective, and the structural properties of substrates, in combination with the arrangement of amino acids within the LBDs, will determine this specificity. Great efforts have been made, with varying degrees of success, to establish the basis for selectivity in drug binding, particularly with respect to P-gp. Through analytical comparison of 100 known P-gp substrate structures, Seelig and colleagues attempted to determine the structural properties of P-gp substrates. A theoretical model was presented that was based on the spatial separation of essential electron-donor groups, and in this model, it was postulated that each hydrophobic substrate must feature two electron-donor groups $2.5 + 0.3$ Å apart, two electron-donor groups $4.6 + 0.6$ Å apart, or three electron-donor groups with the outer two groups separated by $4.6 + 0.6$ Å [52,53].

Alternative methods focused on mapping the drug substrate binding regions of P-gp, using photoaffinity analogs of established P-gp substrates. Such methods revealed several specific binding sites within the LBD. For example, [^{125}I]iodomycin, a derivative of daunorubicin, was found to be bound to amino acids 230–312 (located in TM helices 4 and 5 and the cytoplasmic loop joining them) in isolated hamster P-gp [54]. The prazosin analog [^{125}I]iodoarylazidoprazosin was found to bind to three sites (amino acids 248–312 in TM helices 4–5, 758–800 beyond TMD8, and 1160–1218 in the second NBD) following tricine/PAGE and HPLC separation of ^{125}I-labeled fragments and subsequent Edman sequencing [55]. Similarly, benzophenone analogs of paclitaxel were used to identify two binding sites at amino acids 683–760, which lie in TM helices 7–8, and 985–1088, which begin in TM helix 12 and end after the Walker A motif in the second NBD [56]. Similar studies have identified the binding sites of analogs of azidopine [57], dexniguldipine [58], cyclosporin A [59], and others [60].

Further evidence to indicate that multiple drug binding sites exist within a single LBD was reported following the crystallization of stereoisomeric QZ-59 compounds with mammalian P-gp. A range of distinct residues promoted drug binding, and although it was shown that the same residue may interact with molecules bound at different sites, the specific role of these residues in each interaction differs [27]. Two residues previously identified as being protected by MTS-verapamil labeling and therefore located in verapamil binding sites [61,62] were found also to interact with the QZ-59 compounds. A further 16 residues identified as interacting with the QZ-59

TABLE 5.2 Summary of Pharmacological Substrates of the Three Primary ABC Drug Transporters

Drug	Drug Class/Type	Indication	P-gp	MRP1	MRP2	BCRP
Vincristine	Vinca alkyloid	Cancer	+	+	+33	
Vinblastine			+	+	+ 34	
Doxorubicin	Anthracycline antibiotic		+	+	+ 35	+
Daunorubicin			+	+		+[a]
Paclitaxel	Taxane		+			
Docetaxel			+		+ 36	
Etoposide	Epipodophyllotoxin		+	+	+ 35	+
Teniposide			+	+	+ 37	+
Topotecan	Camptothecin		+	+	+38	+
Irinotecan				+		+
Bisantrene	Anthracene		+			+[a]
Mitoxantrone			+			+
Methotrexate	Folic acid analog			+	+ 39	+
Melphalan	Alkylating agent			+[b]		
Cyclophosphamide				+[b]	+ 40	
Imatinib mesylate	Tyrosine kinase inhibitor		+	+		+
Gefitinib			+	+		+
Flavopiridol	Cyclin-dependant kinase inhibitor					+
Ritonavir	Antiviral	HIV	+	+	+ 41	
Saquinavir			+	+	+ 41	
Nelfinavir			+			
Morphine	Opioid analgesic	Severe pain	+		+ 42	
Fexofenadine	Antihistamine	Seasonal allergic rhinitis. Urticaria	+		+[c] 43	
Cimetidine	H_2-receptor antagonists	Duodenal and Gastric Ulcers	+			
Cyclosporin A	Immunosuppressant	Graft rejection	+		+ 44	

Tacrolimus		Adjunct to liver, kidney, lung, and heart allograft transplantation	+		
Amiodarone	Antiarrhythmics	Severe tachyarrhythmias	+	+[d] 45	
Propafenone			+		
Felbamate	Anticonvulsant	Epilepsy	+		
Topiramate			+	− 46	
Lovastatin	Statin	Hypercholesterolemia	+[e]	− 47	
Simvastatin			+[e]	− 47	
Rosuvastatin				+ 47	+
Pravastatin				+ 48	+
Ondansetron	Antiemetics	Nausea	+		
Digoxin	Cardiac glycoside	Chronic heart failure and atrial fibrillation	+		
Ivermectin	Antinematodal	Onchocerciasis, intestinal strongyloidiasis	+		
Verapamil	Ca^{2+} channel blocker	Hypertension, tachyarrhythmia, angina	+		
Nifedipine			+		
Diltiazem			+		
Trifluoperazine	Phenothiazine antipsychotic	Chronic psychotic disorders, anxiety, and alcoholism	+		
Chlorpromazine		Acute functional psychoses	+		
Trans-flupentixol	Thioxanthene neuroleptic	Chronic schizophrenia	+		
Propranolol	β-Adrenergic blocker	Hypertension, angina	+		

(*continued overleaf*)

TABLE 5.2 (*Continued*)

Drug	Drug Class/Type	Indication	P-gp	MRP1	MRP2	BCRP
Difloxacin	Quinalone antibiotic	Susceptible infections		+		
Erythromycin	Macrolide antibiotic		+		+ 49	
Gramicidin A	Antibiotic		+			
Ciprofloxacin	Fluoroquinolone antibiotic					+
Norfloxacin						+
Dexamethasone	Glucocorticoid	Corticosteroid responsive conditions	+		+[d] 50	
Disulfiram	Alcohol deterrent	Chronic alcoholism	+			

Where information is not from Ref. 5, reference is included in the table. Blank cells indicate no information was obtained. "-" indicates MRP2 was confirmed as having no influence on the drug pharmacokinetics.

[a]Transported by ABCG2R482T mutant only.

[b]Transported as glutathione conjugates.

[c]In mice may be species specific.

[d]Study found ABCC2 transcription and expression increased as a result of drug exposure. Impact on transport is unclear.

[e]Later studies suggest neither drugs are transported by MRP2 47. "+" indicates the drug is transported by the ABC protein.

Source: Adapted from Ref. 5.

compounds were also highlighted for their role in drug binding by an independent study that utilized arginine-scanning mutagenesis to determine residues important in drug translocation. By introducing arginine residues at each position in TM helices 2–12 of a P-gp processing mutant (G251V) and assessing their effect on protein maturation, the location of each residue in relation to possible drug binding sites was established. Several arginine substitutions located on the internal surface of the LBD were shown to reduce verapamil binding affinity 10-fold, while others promoted a 10-fold increase in binding affinity of rhodamine B, again highlighting that multiple binding sites exist within the LBD, promoting the selectivity of P-gp substrates [63].

Some examples of pharmacological agents that are transported by P-gp, MRP1, and BCRP are presented in Table 5.2.

5.3.2 Role of Glutathione in ABCC Subfamily Activity

Despite primarily transporting organic and anionic molecules, MRP1 transports a greater number of substrates than P-gp [64]. This phenomenon has been attributed to the essential role of glutathione (GSH) in drug transport mediated by members of the ABCC subfamily, particularly, MRP1 and MRP2. Although the exact mechanisms by which GSH aids in drug transport remains unclear, evidence suggests that the reducing tripeptide either binds to MRP1 and induces a conformational change to subsequently enhance the transport of hydrophobic compounds or is cotransported with the drugs [31]. Importantly, GSH is required not only for the MRP1-mediated efflux of therapeutics but also for the transport of several physiological substrates, including the well-established substrate of MRP1, leukotriene C_4 (LTC_4) [65]. Other endogenous molecules such as prostaglandin (PG) A_2, 4-hydroxynonenal, and the PGD_2 derivative 15-deoxy-$\Delta^{12,14}$-PGJ_2 are also cotransported with GSH, in each case as GSH-conjugates [65].

GSH itself is a poor substrate of MRP1 and can only be transported in the presence of hydrophobic compounds. For example, vincristine, a vinca alkaloid used in the treatment of various cancers, cannot be transported by MRP1 alone. In the presence of GSH, however, both vincristine and GSH accumulate in vesicles expressing the transporter, indicating that GSH is cotransported with the drug [66] in a mechanism where both substrates rely on each other for transport. Studies using inside-out membrane vesicles isolated from MRP1-overexpressing cells indicate that without physiological concentrations of GSH present, MRP1 lacks the ability to transport many unmodified anticancer drugs [67,68]. Unlike the endogenous substrates of MRP1, however, there is no evidence to indicate that conjugates form between GSH and xenobiotic substrates. Instead it is believed that these agents are effluxed together with GSH in a simple cotransport mechanism or following conformational changes to MRP1, they are induced when GSH and the drug bind to the protein. Currently, little is known about the mechanism by which GSH and substrates induce conformational changes to MRP1. However, several studies have been carried out to determine possible substrate–GSH binding sites. Photoaffinity labeling techniques using derivatives of MRP1 substrates such as [H_3]LTC_4, iodoaryl-azido GSH (IAAGSH), and azidophenacyl GSH (APAGSH) have predicted the location of various substrate binding sites within the protein [69]. In the case of [H_3]LTC_4, it was suggested that substrate binding sites may exist within the lipid bilayer, possibly in the final two TM segments of TMD1 and TMD2 [69]. Site-directed mutagenesis studies have also revealed several sites in the TMDs of MRP1 that

may be important in the binding of GSH, such as Lys332 and His335 in α-helix 6 of TMD2 [70,71]. Using homology models to explore the influence of a range of proline substitutions in various TM α-helices of MRP1, it has also been shown that mutating Pro478 of TM9 to an alanine residue selectively reduces LTC_4 transport [72]. Similar studies aimed at determining substrate binding sites of MRP1 inhibitors showed preferential cross-linking of the radiolabeled photoactive compounds to segments within the third (COOH-proximal) TMD [73,74], suggesting that MRP1 has more than one drug binding site. It is also possible that MRP1 substrates such as GSH form individual, yet not necessarily unique, binding sites within a common binding pocket in the transporter [65].

Like MRP1, the mechanisms by which GSH facilitates MRP2-mediated drug transport is unclear. Early studies revealed GSH-stimulated vinblastine transport by rabbit MRP2 expressed in the insect cell line Sf9 [75]. Similarly a 10-fold increase in GSH was observed in the canine cell line MDCKII transfected with MRP2 versus wild-type cells, leading to the conclusion that MRP2 facilitates a low affinity transport of GSH [76]. Subsequent studies using MDCKII reported similar findings in the transport of vinblastine [34]. This study also found GSH to be cotransported with low concentrations of sulfinpyrazone; however, at high concentrations of the drug, transport of GSH was not observed, while transport of sulfinpyrazone continued [34]. It was hypothesized that two binding sites exist in MRP2, and at high concentrations, sulfinpyrazone will occupy both sites, preventing the cotransport of GSH, yet allowing sulfinpyrazone to be transported alone [34].

5.3.3 Influence of ABC Transporters on Drug Accumulation in Vesicles

Several ABC drug transporters localize to the lipid bilayer membranes of intracellular vesicles such as lysosomes and endosomes. From these locations, the drug transporters facilitate the active sequestration of various agents into the acidic compartments for drug efflux or redistribution away from the target. It was demonstrated that the ABC transport pump, ABCA3, localized to the lipid bilayers of lysosomes and multivesicular bodies where it conferred resistance to a range of cytotoxic agents including daunorubicin, mitoxantrone, etoposide, and vincristine. This drug resistance resulted from an ABCA3-mediated drug uptake into vesicles of low pH [12]. Earlier studies had also implicated the ABC transport proteins in the uptake of anticancer drugs into acidic vesicles of the membrane trafficking system. In 2003, Rajagopal and Simon provided evidence that in HeLa cells, MRP1, P-gp, and BCRP were not only localized to membranes of intracellular vesicles but their roles at these sites contributed to drug resistance phenotypes. Using a cell impermeable MRP1 inhibitor, it was highlighted that two separate pools of MRP1 were active within the cell and active sequestration of the drug by vesicular MRP1 alone was sufficient to confer drug resistance. In this study, doxorubicin was shown to colocalize in lysosomes with MRP1 and accumulation of the drug was prevented following pretreatment with verapamil, an ABC transport pump inhibitor [13,77]. In a separate study using HL-60 cells, it was reported that while the anthracycline antibiotic, doxorubicin, accumulated in acidic vesicles following passive diffusion of the lipid bilayer, and subsequent protonation of the compound, the zwitterionic compound, sulforhodamine 101, accumulated in the Golgi body following an MRP1-mediated uptake into the organelle [77].

5.4 INFLUENCE OF ABC ACTIVITY IN DRUG RESISTANCE

Although the ABC proteins have been shown to transport many therapeutic agents, with the cause of MDR being multifactorial, their influence on this phenomenon, especially *in vivo*, remains debatable. In a review of P-gp function in cancer, Ambudkar and colleagues suggested five points that should be met in order to declare an ABC transport protein responsible for drug resistance in a clinical setting. First, the protein should be expressed at a level comparable to that which is known, *in vitro*, to confer resistance. Second, this level should predict the degree of resistance, while in the third point, it was suggested that any increase in drug resistance during treatment should occur together with an increase in protein expression. The fourth point suggested that if the ABC protein is the sole contributor to resistance, then this resistance should be reversed with protein inhibition. Finally, it was suggested that any inhibitor would be advantageous as an adjuvant therapy [60].

To date, a total of 18 ABC transport proteins have been implicated in drug resistance [78]. Of these 18 proteins, only P-gp, MRP1, and BCRP have been identified on several occasions to contribute to the development of the MDR phenotype *in vivo*, and hence, greater attention will be paid to the role of these proteins in drug resistance. Although these proteins may confer resistance to drugs used in the treatment of many diseases, their influence on the efficacy of cancer therapy dominates the literature. As such, this chapter focuses on the role of the ABC proteins in cancer drug resistance.

5.4.1 ABCB1 (P-gp) and Cancer Drug Resistance

A large amount of clinical data correlates expression and function of P-gp with response to therapy in cancer patients. Two independent studies have drawn correlations between expression level of the protein and response to therapy in the treatment of breast cancer. One of these studies indicated that the presence of P-gp, which was increased 1.8-fold by prior exposure to chemotherapy, translated to a three- to fourfold greater chance of treatment failure [79], while the other reported a strong correlation between the extent of taxol and doxorubicin resistance in 359 resected breast cancer samples and the expression levels of P-gp [80]. Similar results were observed in nonsmall-cell lung carcinoma (NSCLC) where 68% of tumors that expressed P-gp were resistant to the drug paclitaxel, while those lacking the protein responded well to therapy [81]. In contrast, P-gp was overexpressed in only 2% of 67 metastatic glioma patients despite a high level of drug resistance observed in all patients. In this case, it was suggested that P-gp activity is just one mechanism contributing to the MDR phenotype [82]. Abe and colleagues supported these findings in 1998 reporting that only 4–23% of gliomas were P-gp positive. This study also highlighted that P-gp expression was higher in patients who had previously been exposed to chemotherapy, suggesting the protein played a role in acquired drug resistance [83]. P-gp has also been identified as a cause of drug resistance in leukemias and lymphomas, where expression of the protein is upregulated after exposure to chemotherapy. It is expressed in ~30% of all acute lymphoblastic leukemia (ALL) patients at diagnosis, and although studies have reported an association between its expression and patient response to therapy [84,85], these findings were contradicted by other studies [86–88].

5.4.2 ABCC1 (MRP1) and Cancer Drug Resistance

MRP1 is ubiquitously expressed throughout the body and as a result high levels of the protein have been identified in most cancer types. Unlike P-gp, MRP1 does not appear to contribute to MDR to any significant degree in metastatic breast cancer nor ALL. Despite this, MRP1 is associated with the development of MDR in a large number of tumor types including lung carcinomas and gliomas. The protein is regularly expressed in NSCLC where it correlates highly with a poor patient response to chemotherapy and overall survival [64,89]. These findings were described in a study that assessed MRP1 levels in 126 samples via immunohistochemistry. In this study, MRP1 levels were more highly expressed in stage 1 tumors versus late-stage tumors and were particularly high in patients who had not yet received chemotherapy, suggesting that the overexpression is inherent in NSCLC and not induced by chemotherapy exposure. It was also highlighted in the same study that although MRP1-positive tumors appear less aggressive, they are particularly resistant to chemotherapy [89]. Similar results have been reported in small-cell lung carcinoma (SCLC) where MRP1 expression is also elevated in patients who had a relapse [90]. Benyahia *et al*. reported that MRP1 was highly expressed in the vascular endothelial cells in 93% (14/15) of the glioma tumor samples examined. From this localization, it was proposed that MRP1 limits the uptake of drug into the brain tumor cells [91]. The protein was also found to be expressed along with MRP4 and MRP5 to the luminal side of brain capillary endothelial cells where again, it was proposed to influence drug penetration of the blood brain barrier [92]. The effect of MRP1 expression in human gliomas was also discussed in 2005 when a high level of expression was shown in 51% of the human glioma specimens and primary cell cultures examined. No difference between primary and recurrent glioma was observed, and it was subsequently concluded that MRP1-mediated drug resistance is intrinsic to glioma tumors [93].

5.4.3 ABCC2 (MRP2) and Cancer Drug Resistance

The effect of MRP2 on *in vivo* cancer drug resistance remains to be defined. However, it is clear that many agents used in the treatment of cancers are transported by the pump in *in vitro* studies. MRP2 was found to confer resistance to a large range of compounds including cisplatin and methotrexate. This resistance was highlighted after cisplatin resistance was successfully reversed using anti-MRP2 hammerhead ribozymes in the ovarian carcinoma cell line A2780RCIS [39]. MRP2-facilitated methotrexate resistance was also highlighted in an ovarian carcinoma cell line, with resistance observed following short-term exposure (1–4 h) of the drug [94]. Although overexpression of MRP2 has been shown to confer resistance to the tyrosine kinase inhibitor sorafenib in LLC-PK1 cells [95], earlier studies suggest that this is not common to all drugs of this class, with no active transport of erlotinib observed in MDCKII cells stably expressing MRP2 [96]. Other studies, using cell lines stably transfected with human MRP2, reveal that this protein confers resistance to etoposide, cisplatin, doxorubicin, and epirubicin. This resistance was up to 10-fold in the case of cisplatin and fourfold in the case of etoposide [35]. These results were supported in a study of drug resistance in esophageal squamous cell carcinoma (ESCC) treatment, which found that inhibition of MRP2 expression by siRNA reduced resistance to the 5-fluorouracil, doxorubicin, and cisplatin treatment regime [97]. Although *in vitro* studies had shown MRP2 to transport docetaxel, *in vivo* studies using MRP2 knockout mice revealed no difference in

drug pharmacokinetics compared to wild-type mice. When CYP3A and P-gp were also knocked out, however, (Cyp3a/Mdr1a/b/Mrp2-/-) changes in the drug's pharmacokinetics were observed. These changes were evident when compared to both wild-type mice and Cyp3a/Mdr1a/b knockout mice proficient in MRP2, indicating MRP2 will reduce docetaxel exposure in the absence of both CYP3A and Mdr11/b [36].

5.4.4 ABCG2 (BCRP) and Cancer Drug Resistance

BCRP was originally identified by Doyle and colleagues [98] in 1998 in the MC7-AdrVp breast cancer cell line. Examination of BCRP and its role in drug resistance in leukemia patients has yielded a variety of results. In a study of 46 mixed lineage ALL samples, it was determined that BCRP was not only more highly expressed in the B-cell lineage compared to the T-cell lineage but also had a greater influence on MDR in these cells [99]. BCRP was also implicated in mitoxantrone efflux in 70% (21/30) of untreated ALL patients despite low mRNA levels being detected [100]. Furthermore, BCRP levels were found to be 10-fold higher in acute myeloid leukemia (AML) patients who did not achieve remission compared to those patients who responded well to therapy [101]. However, contradictory to these findings were reports that BCRP does not correlate to drug resistance in leukemia patients. One such report examining Ara-C resistance in 13 infants and 13 noninfants found that despite *ABCG2* mRNA expression being positively correlated to Ara-C resistance, the drug was not a substrate of the transporter and hence other mechanisms were responsible for this resistance [102]. Sauerbrey and colleagues also reported that although BCRP expression was lower in the T-cell lineage, there was no significant correlation between protein expression levels and response to therapy [103]. Similarly there are mixed reports on the influence of BCRP in drug resistance in solid tumors. In a recent study it was found that although there was no significant association between P-gp and MRP1 expression in SCLC patients' response to therapy and survival, there was a significant association between BCRP expression and tumor response to chemotherapy and progression-free survival [104].

5.4.5 Impact of Other ABC Transporters on MDR

Although there are few reports of the remaining ABC proteins influencing MDR *in vivo*, a substantial number of *in vitro* studies have provided evidence that implicates various ABC transport proteins in the onset of MDR. It was found that ABCA2 confers resistance to mitoxantrone in the BCRP1-negative GLC4-MITO cell line when compared to the GLC4 cell line [105], and it has also been established that the same protein confers resistance to the estrogen derivative, estramustine [106]. As previously mentioned, ABCA3 reportedly conferred resistance in leukemia cells to the anthracycline antibiotics, etoposide and vincristine, when localized to the lipid bilayer membranes of acidic vesicles [12]. This finding supported an earlier report that identified ABCA3 overexpression in pediatric AML using microarray technology. With a median expression level of threefold higher in patients who had failed to achieve remission following the first round of chemotherapy, it was suggested that ABCA3 conferred resistance to the anthracycline antibiotics in AML patients [107].

A greater number of ABCB members have been implicated in cancer drug resistance. Along with P-gp, both ABCB2 and ABCB3 have been shown to contribute

to mitoxantrone and etoposide/teniposide resistance [7,78], while both ABCB4 and ABCB11 have been implicated in resistance to paclitaxel [8]. ABCB5 has been shown to confer resistance to doxorubicin, camptothecin, and 5-flurouracil [108]. One study that flagged ABCB5 as a contributor to doxorubicin resistance in malignant melanoma highlighted exclusive expression in a subpopulation of tumor stem cells. Blockade of the ABCB5 transporter using a specific inhibitory monoclonal antibody reversed doxorubicin resistance in G3361 melanoma cells, and the hypothesis was raised that targeting this transporter in an adjuvant therapy may enhance the efficacy of those agents used in the treatment of malignant melanoma [109]. The final member from the ABCB subfamily to be implicated in drug resistance is ABCB6 that has been shown *in vitro* to confer resistance to both cisplatin and camptothecin [78,110].

Like the ABCB subclass, a large number of ABCC subclass members have been shown to confer resistance to various anticancer agents. While MRP1 and MRP2 remain the most actively researched members of this subclass, ABCC3, ABCC4, ABCC5, ABCC6, ABCC10, and ABCC11 have all been shown to influence drug resistance phenotypes [78]. ABCC3 confers only a mild resistance to the vinca alkaloids, epipodophyllotoxins, methotrexate, and cisplatin [37]. Although the protein is able to transport organic compounds conjugated to GSH, experiments assessing GSH efflux suggested that ABCC3 cannot transport the tripeptide alone and does not require it for drug transport [37]. Evidence implicating ABCC4 in MDR phenotypes remains limited, although it has been suggested to be a possible target for cancer therapy. This is due to its role in the regulation of the intracellular concentrations of cAMP and the subsequent maturation of leukemic cells [111]. A report in 2005 concluded the transporter contributes to neuroblastoma prognosis through its activity in effluxing irinotecan and its active metabolite SN38 [112]. However, a later study assessing the systemic clearance of these agents in mice determined that ABCC4 did not influence their elimination, while P-gp did [113]. *In vitro* studies implicated ABCC4 in the resistance of cyclophosphamide and ifosfamide [114], topotecan and methotrexate [115], as well as camptothecin, SN-38, and other camptothecin derivatives [116] in HepG2 and HEK293. A larger number of substrates have been reported to be transported by ABCC5, yet it has also been highlighted that this transport is unlikely to contribute significantly to clinical MDR [117]. Various studies, each utilizing cells transfected with MRP5, have suggested a role for the protein in the transport of cisplatin, Oxaliplatin doxorubicin [118], etoposide, and teniposide [119]. Like many of the other members of the ABCC subclass, ABCC6, otherwise known as *MRP6*, has been shown to confer a low level of resistance to the anthracyclines, epipodophyllotoxins, and cisplatin [120]. Evidence to indicate this is, however, limited, and the suggestion has been made that like MRP5, MRP6 makes little contribution to clinical MDR [78]. After ruling out the influence of P-gp, BCRP, and various members of the ABCC family of drug transporters, it was recently shown that expression of ABCC10 (MRP7) confers resistance to vinorelbine in NSCLC cell lines [121]. This protein was also implicated in taxane resistance in transfected HEK293 cells where cellular accumulation of radiolabeled paclitaxel was reduced compared to cells transfected with a control plasmid [122]. The final member of the ABCC subclass that has been implicated in drug resistance is ABCC11. This protein was shown to confer resistance to 5-flurouracil in a resistant subline of the PC-6 human SCLC cell line. In this case, resistance facilitated by MRP8 was reported at up to 25-fold [123].

5.5 INFLUENCE ON ADVERSE REACTIONS AND DRUG–DRUG INTERACTIONS

Drug–drug interactions (DDIs) manifest themselves clinically through altered pharmacokinetics, where one drug influences the absorption or disposition of another, and through altered pharmacodynamics, where drugs used in combination may produce additive, synergistic, or antagonistic effects [124]. Direct clinical evidence of P-gp inducing DDI is limited due to cross-specificity of P-gp substrates with the drug-metabolizing enzyme CYP3A4. While much of the current evidence indicates that CYP3A4 is a greater contributor to DDIs [124,125], it has been proposed that the ABC proteins contribute to DDI, and subsequent adverse side effects, following coadministration of antiretroviral agents used in the treatment of HIV with antifungals, antibiotics, cardiovascular agents, and recreational/illicit agents such as cocaine, lysergic acid diethylamide (LSD), marijuana, and 3,4-methylenedioxymethamphetamine (MDMA) [124].

While P-pg may not define drug disposition *in vivo* alone, its contribution to peak plasma concentrations and elimination rates of substrates with narrow therapeutic windows may be great enough to produce severe adverse reactions. This is the case with the cardiovascular drug digoxin, which is not metabolized by the cytochrome P450 family of enzymes but is a substrate of P-gp. Itraconazole, an antifungal agent that is known to modulate the function of P-gp, increases the plasma concentrations of digoxin while decreasing its renal clearance [126]. Similarly, the calcium channel blocker mibefradil was removed from the market as it increased the C_{max} and AUC of digoxin to levels that induced severe adverse reactions. [127]. P-gp was also implicated in DDIs between digoxin and rifampicin, an antibiotic used in the treatment of tuberculosis. In this case, the AUC of digoxin administered orally was significantly reduced when coadministered with rifampicin. This was attributed to a rifampicin-induced increase in the intestinal expression of P-gp, preventing the absorption of digoxin in the gut [128]. It should be noted, however, that although many studies have implicated digoxin in P-gp-mediated DDIs, a recent retrospective analysis of 123 such studies concluded that the majority of reported DDIs involving digoxin were not clinically significant [125].

The influence of P-gp on interactions between dopaminergic agonists used in the treatment of Parkinson's disease has also been assessed. While each of the agents examined were P-gp substrates, only bromocriptine inhibited substrate transport. The subsequent effect of this inhibition was evident on the intracellular transport of levodopa (L-dopa), which increased over twofold. With L-dopa, bromocriptine, and other dopaminergic agonists administered simultaneously in the treatment of Parkinson's disease, it was proposed that through its effect on P-gp function, bromocriptine could alter the disposition of L-dopa and subsequently induce an adverse effect as neurotoxicity [129].

5.6 MODULATION OF ABC PROTEIN FUNCTION

5.6.1 Molecular Mechanisms to Modulate ABC Function

Several molecular techniques have been adopted in an attempt to inhibit the function of the ABC drug transporters. P-gp targeting hammerhead ribozymes delivered via liposomes were utilized to achieve P-gp inhibition and reversal of vincristine resistance

in the breast cancer cell line MCF-7/R. This study, however, failed to achieve similar results in the MOLT-3/TMQ_{800} cell line [130]. Successful inhibition of P-gp function in MOLT-3/TMQ_{800} cell line was achieved, however, following transduction of MDR-1 hammerhead ribozymes into the cells. A retroviral vector containing Pol III was utilized to improve ribozyme activity and resulted in decreased levels of P-gp, along with an increase in vincristine sensitivity. On the basis of these results, it was suggested that the ribozyme targeting the translation initiation site was the most efficacious in reversing drug resistance [131].

Currently, gene silencing via the use of short siRNAs targeted toward the ABC transport proteins is proving successful in the reversal of drug resistance in *in vitro* studies. The efficacy of siRNAs targeted toward the *ABCB1* gene was revealed using the multidrug-resistant human pancreatic carcinoma and gastric carcinoma cell lines EPP85-181RDB and EPG85-257RDB. Expression of P-gp was reduced 91% at the mRNA and protein levels and this was correlated to significant improvements in the efficacy of daunorubicin. This reduction in drug resistance was reportedly at a level of 89% in EPP85-181RDB cells and 58% in EPG85-257RDB cells [132]. Successful inhibition of P-gp translation was also reported by Stege and colleagues in 2004. A complete reversal of resistance was achieved in the EPG85-257RDB cell line following exposure to an anti-P-gp short-hairpin RNA expression vector. This stable reversal of resistance was reportedly associated with a significant increase in intracellular accumulation of the anthracycline antibiotic daunorubicin [133].

5.6.2 ABC Modulators as Adjuvant Therapies

A large number of small molecules have been developed over the years, which modulate or inhibit the activity of the ABC transport proteins. Many of these molecules have entered clinical trials as adjuvant therapies, particularly in the treatment of cancer; however, the majority were not found to be clinically relevant, most often due to excessive adverse effects and toxicities at doses well below those required to inhibit the ABC proteins. These small molecules modulate the function of the ABC transport proteins through a number of mechanisms including competition at the drug substrate binding pocket, binding to high affinity sites in the NBD or through unique mechanisms.

Verapamil, cyclosporin A, and the phenothiazines, which act as competitors at the substrate binding site, are first-generation ABC inhibitors that were considered as adjuvant therapies for cancer treatment and they subsequently entered clinical trial. These compounds are associated with severe dose-limiting side effects that include cardiomyopathy, hypotension, and increased toxicity of the cytotoxic compounds. In the case of calcium channel blockers such as verapamil, adverse cardiovascular effects occur at dose concentrations lower than that required to inhibit the ABC transporters [134–136]. Clinical trials involving these first-generation inhibitors of ABC transporters indicated that although they may improve the efficacy of treatment in leukemia patients [137,138], the benefit in the treatment of solid tumors remains debatable. Early studies indicated that no advantage was gained in solid tumors, predominately due to the onset of cardiotoxicity at doses required to inhibit P-gp [139,140]. More recent studies, however, have reported that inclusion of verapamil in the treatment of doxorubicin-resistant metastatic breast cancer improved patient response without the onset of dose-limiting side effects [141].

The second-generation compounds dexverapamil and PSC833, which are analogs of verapamil and cyclosporin A, respectively, are associated with fewer and less intense

adverse effects. Their success in clinical trial, however, remains limited. Dexverapamil was assessed in combination with doxorubicin and epirubicin in patients with metastatic breast cancer, and although it was reported that inclusion of dexverapamil did not intensify anthracycline-associated cardiotoxicity, the number of patients achieving a complete response to therapy were limited [142,143]. Although the results obtained in these studies were considered promising, it could not be confirmed that this was a consequence of ABC transport inhibition. These conclusions were again reflected in another phase II trial in patients with advanced pancreatic cancer. The *in vivo* efficacy of PSC833 was assessed in combination with cytarabine, mitoxantrone, and etoposide in patients with AML. One study reported an improved efficacy and higher frequency of complete response (29%) compared to traditional response rates [144], and these results were supported by a separate study that reported a complete response rate of 26% in AML patients receiving the same combination of drugs [145]. Despite these positive results, it was also reported that excessive neurological side effects occur at doses higher than 10 mg/kg [146], and owing to a combination of poor response rates and excessive adverse reactions, one clinical trial was terminated prematurely [147]

Several newer "third-generation" compounds have been designed to specifically inhibit the function of P-gp. One agent known as *tariquidar* (XR9576) finds its mode of action in a noncompetitive mechanism, binding to P-gp at a site alternative to that of the transport substrate [148]. This compound is characterized by a greater selectivity toward P-gp and a slower dissociation rate from the protein. *In vitro* assessment of the agent suggested that P-gp inhibition may last up to 22 h after the drug is removed from cells [149]. Despite this high selectivity and potency, it was reported that tariquidar possessed limited clinical efficacy when used in combination with the anthracyclines and taxanes in patients with drug-resistant, advanced breast carcinoma [150].

Another third-generation P-gp inhibitor is LY335979. This agent shows no inhibition of BCRP or MRP1 and unlike tariquidar, competes with other P-gp substrates for binding at the substrate binding site [151,152]. Initial phase I clinical trials in patients with advanced malignancies reported several dose-limiting adverse reactions including neurotoxicity, cerebral dysfunction, hallucinations, and palinopsia [153]. A later study reported that a 640 mg/m^2 dose of LY335979 could be safely administered over 48 h intravenously. This study assessed the efficacy of the compound in combination with doxorubicin and reported that no dose-limiting side effects occurred [154]. Similarly, during a phase II clinical trial in patients with untreated nonHodgkin's lymphoma, LY335979 was safely administered over a 24-h schedule without enhancement of cytotoxic related toxicities [155].

5.7 TRANSCRIPTIONAL REGULATION OF THE ABC GENES

In keeping with the focus of this chapter, the transcriptional regulation of *ABCB1, ABCC1*, and *ABCG2* in response to the presence of small molecules is discussed. It is important to remember, however, that with many of the ABC proteins having alternative physiological functions and cellular/organ locations, their transcriptional regulation may be induced and maintained by molecular mechanisms not discussed in this chapter.

The constitutive expression of each ABC drug transport protein is dictated by a complex network of transcription factors that interact with each other and response

elements within ABC gene promoter regions. Each of the ABC genes identified to date contains TATA-less promoter regions and possesses initiator elements to which various transcription factors bind before the recruitment of RNA polymerase II [156]. The *ABCB1* gene is known to possess an initiator sequence −6 to +11 nucleotides surrounding the transcription start site [157] as well as an inverted CCAAT box (−79 to −75) and a GC-rich element (−56 to −43). The latter two DNA sequence regions are among the most common polymerase II promoter elements and interact with the transcription factors NF-Y and Sp1/Sp3, respectively [158,159]. An additional GC element is known to reside upstream within the promoter region (−110 to −103) that does not interact with Sp1 but may interact with another member of the Sp transcription factor family.

Like *ABCB1*, GC-rich sequences play a vital role in the recruitment of the transcription machinery to the *ABCC1* and *ABCG2* genes. In the case of *ABCC1*, CG elements that lie −91 to +103 nucleotides adjacent to the transcription start site have been shown to interact with Sp1 [160], while in the case of *ABCG2*, several potential Sp1 sites were identified within sequences 300 bp upstream of the transcription start site [161]. There are several proteins that have been reported to interact with response elements within the ABC gene promoter regions to either induce or suppress transcription of the ABC drug transporters. These include p53, Ras/Raf, AP-1, and the Wilms' tumor suppressor protein [162]. Of greatest relevance to the pharmacology of small molecules, however, is the overexpression of the ABC drug transport proteins in response to drug exposure.

Several nuclear receptors, including steroid and xenobiotic receptor (SXR), retinoic acid receptor (RAR), farnesoid receptor (FXR), and constitutive androstane receptor (CAR), are known to influence transcription of the ABC genes in response to various xenobiotics [162]. SXR [also known as *pregnane X receptor* (PXR) in rodents, PAR and NR1I2] is activated by a range of small molecules. Xenobiotic detection and subsequent activation of the nuclear receptor involves a large and flexible hydrophobic pocket that resides in the N-terminal LBD. The PXR ligand binding cavity, unlike that of other nuclear receptors, senses a large range of molecules, serving a general protection mechanism against toxic substances [163]. Following activation, the nuclear receptor forms a heterodimer with the retinoid X receptor α (RXRα). This interaction, which is believed to be facilitated by two activation factor domains that lie within the LBD [164], is common among all nuclear receptors involved in the transcriptional regulation of the ABC drug transporters and precedes binding of the heterodimer to response elements within the promoter region of the ABC genes [164]. Binding of the heterodimer to DNA is mediated by a highly conserved region known as the *DNA-binding domain* (DBD). Both the PXR–RXRα and CAR–RXRα heterodimers can bind to the same response elements within the enhancer regions of the *ABCB1* promoter.

5.8 POLYMORPHISMS—EFFECT ON GENE EXPRESSION AND PROTEIN FUNCTION

5.8.1 Polymorphisms in ABC Transport Protein Genes

The growing desire for therapeutics tailored to the individual has, in recent years, stimulated much research into determining the effect of polymorphisms on the function and

transcriptional regulation of the ABC transport proteins. SNPs, which make up over 90% of all genetic mutations, are believed to contribute significantly to the interindividual differences of drug response [165]. This is often revealed through diminished drug efficacy and increased adverse reactions.

SNPs, which are defined as *single nucleotide variations* that exist in 0.1% of the population or greater, may be classified as synonymous or nonsynonymous. Synonymous SNPs are those that do not influence the tertiary structure of the protein but may play a role in the transcriptional regulation of the gene in which it resides. Nonsynonymous SNPs, on the other hand, produce an amino acid change in the protein sequence and hence may affect protein function, interaction with other proteins, or even folding of the tertiary structure. Predicting associations between SNPs and a particular disease, drug response, protein structure or function is made difficult by the number of SNPs believed to exist throughout the genome. This number is rapidly growing, and currently, over 6900 SNPs have been reported in 12 of the major ABC genes [166]. Owing to a phenomenon in which alleles in close proximity are often inherited together, strong correlations may be identified in the expression of minor alleles of different genomic regions. Termed *linkage disequilibrium* (LD), this redundancy of polymorphisms may be exploited in various ways to permit efficient detection of potentially functional SNPs and determine their association with disease and protein function [167].

One method that is commonly adopted in SNP association studies is the selection of a smaller proportion of SNPs, known as *tagging SNPs* (tSNPs), to represent most of the genetic variation within a particular region of interest [167,168]. These tSNPs must not only be in LD but it must also be assumed that no chromosomal recombination has occurred within the region to disrupt the SNP haplotype that would otherwise exist. tSNPs may be selected based on known haplotype distribution or pairwise r^2 LD, the latter being used primarily to select SNPs that best represent different LD blocks within a gene [167,169]. While each method has been shown to select tSNPs with similar predictive power, studies aimed at comparing the effectiveness of each technique suggest those based on haplotype information are most efficient, allowing fewer SNPs to be selected for the same predictive power [169]. Selection of tSNPs may not, however, represent other SNPs in regions of low LD. Similarly, an SNP in a genomic region that is under recent positive selection pressure is generally observed at a higher frequency and is rarely represented by other SNPs in the same region because of differences in ancestral history [166].

Signatures of recent positive selection have been implicated to permit the prediction of potentially functional SNPs. The SNP alleles e22/2677 T (rs2032582) and e27/3435 T (rs1045642) within the *ABCB1* (*MDR1*) gene were previously associated with various functional differences and differences in drug response/other phenotypes and were found to display signatures of recent positive selection in the Chinese population [170,171]. These two positively selected *ABCB1* SNP alleles were found to protect Chinese but not Caucasian men from late-onset (> 60 years) Parkinson's disease [172–174]. The signature of recent positive selection was later employed to demonstrate the feasibility of identifying a previously unknown potentially functionally significant SNP at the *ABCC1* gene locus [175]. The major G allele of SNP 5′UR/G-260C (rs504348) at the promoter region of the *ABCC1* gene was found to be under recent positive selection and to mediate lower *ABCC1* promoter activity compared to the C allele [175]. Before the identification of this potentially functionally significant SNP allele at the *ABCC1* gene that is under recent positive selection, researchers have

failed to detect associations between various SNPs at the *ABCC1* gene and functional differences [176–179]. One possible reason for their failure to detect an association between SNPs at the *ABCC* gene locus and functional difference is due to the general low LD observed at the *ABCC1* gene locus and that this potentially functionally significant promoter SNP under recent positive selection at the *ABCC1* gene locus was never examined in any of the studies. Signatures of recent positive selection were then employed to identify 32 SNPs of potential functional significance at 18 ABC gene loci that are known or implicated to transport drugs [171]. As discussed in this study, it may be assumed that positively selected genomic regions have benefitted the survival of the species in recent evolutionary times and hence play an important functional role. In the situation where an induced change to protein function aids survival, the SNP will be maintained within a population, while those SNPs that produce detrimental effects will reduce in frequency [171].

5.8.2 Effect of SNPs on the Function, Structure, and Expression of ABCB1 (P-gp)

The earliest SNPs believed to affect the expression of P-gp were identified using osteosarcoma cells in 1994. Two SNPs in the promoter region of *ABCB1* gene were identified and the 5′UR/T+103G point mutation was found to enhance the promoter activity of *ABCB1* in cells exposed to the anticancer drugs, doxorubicin and vincristine [180]. Since these early discoveries, advances in high throughput DNA sequencing techniques together with the popularity of this gene in the field of pharmacogenetics have seen a substantial number of additional polymorphisms identified. In the latest release of NCBI's SNP database (dbSNP build 130), 62 coding region SNPs were reported at the *ABCB1* locus. Of these SNPs 40 are nonsynonymous; and 22, synonymous. The majority of these SNPs have yet to be genotyped in any major population cohort, and many of the coding region SNPs associated with *ABCB1* are expressed at low frequency in each of the populations assessed HapMap (phase III) (http://www.hapmap.org/).

Nonetheless, three high frequency SNPs at the *ABCB1* gene locus, namely, e13/C1236T (rs1128503), e22/G2677T/A (rs2032582), and e27/C3435T (rs1045642), are often found to be in strong LD, having been identified as part of many haplotypes where the minor alleles may be expressed at highly variable frequencies in different populations [170,181–185]. Although a number of studies have drawn correlation between the presence of these SNPs and either drug response or disease progression, it remains difficult to determine which are causative and which are merely inherited with causative SNPs, which is a confounding issue of the haplotype phenomenon. Despite a general consensus that synonymous SNPs play insignificant roles in the function and expression of P-gp, a recent hypothesis has suggested that the minor T allele at the e27/C3435T (rs1045642) locus results in a codon that is rarely used in humans. This rare codon is hypothesized to result in ribosomal stalling during translation, which affects not only the rate of translation but also the chaperone-facilitated protein folding [186,187]. Early studies into the function of the synonymous SNP e27/C3435T (rs1045642) reported its association with decreased duodenal expression of P-gp and subsequent increase in the plasma levels of the cardiac glycoside digoxin [188]. However, these results could not be replicated by other studies that found either no influence on mRNA expression [189] or an increased expression of the protein in the

duodenum [190]. In a similar fashion, the influence this SNP has on drug activity is controversial. While expression of the minor e27/3435 C allele was correlated with increased efflux of the P-gp substrate, rhodamine 123 [191], it was associated with reduced efflux of nelfinavir [192] and no effect on fexofenadine efflux [193]. A few reports have implicated the wild-type e27/3435 C allele on drug response. Plasma levels of atazanavir, for example, were determined to be higher in HIV patients expressing the CC genotype [194], while the same genotype was linked to a greater incidence of steroid side effects in kidney transplant patients compared to the TT minor allele genotype [195].

Evidence regarding the influence of the nonsynonymous SNP e22/G2677T/A (rs2032582) on the function of P-gp is equally inconclusive. Again, many of the studies exploring the influence of this SNP on *in vivo* P-gp function have assessed this SNP in combination with other SNPs as a haplotype. Functional validation conducted *in vitro* has shown that expression of Ala893, which is produced by the minor 2677 T allele, was found to confer a reduced level of digoxin efflux compared to cells expressing the Ser893 variant [182]. This finding supported an *in vivo* assessment of fexofenadine disposition, where a reduced oral bioavailability of the drug was associated with expression of Ser893, suggesting an enhancement of P-gp activity. It was, however, noted that in most of these studies, the E22/G2677T/A (rs2032582) SNP was studied together with the E27/C3435T (rs1045642) SNP as a haplotype, making it difficult to determine which SNP exerts the greatest influence [182]. The 2677TT/3435CC genotype combination was also associated with lower plasma concentrations of fexofenadine compared to other genotype combinations involving these SNPs [196], while the 2677TT genotype was positively correlated with a higher risk of CsA failure in steroid-resistant ulcerative colitis [197]. e22/G2677T/A (rs2032582) SNP was also positively correlated to tacrolimus neurotoxicity [198], increased resistance to antiepileptic drugs [199], and increased response to cytarabine in AML patients [200]. Curiously, some studies have found no association between e22/G2677T/A (rs2032582), either individually or in haplotype with other SNPs, and drug response [185,201]. For example, e22/G2677T/A (rs2032582) did not influence plasma trough concentrations of ritonavir in HIV patients [202], rhodamine 123 efflux in peripheral blood lymphocytes [203], tacrolimus pharmacokinetics in renal transplant patients [204], or blood, semen and saliva concentrations of ritonavir or lopinavir in HIV patients [205].

The third coding SNP that occurs at highly variable frequencies in different populations and in strong LD with the e22/G2677T/A (rs2032582) and e27/C3435T (rs1045642) SNPs is the e13/C1236T (rs1128503) SNP. This SNP, being synonymous, does not change the amino acid Gly412. Nonetheless, some studies have indicated that this SNP may influence drug response. Significantly increased exposure to irinotecan was associated with the 1236TT genotype [177], whereas the 1236CC genotype was associated with increased response to temozolomide treatment in glioblastoma patients [206]. Positive correlation has also been drawn between this SNP and patient response to CsA [207] and nelfinavir [192]. Nonetheless, some studies also indicated that this SNP does not influence drug response. For example, response to both lopinavir and ritonavir was shown not to be affected by this SNP in HIV patients [205].

Aside from these three commonly reported SNPs, several other SNPs at the *ABCB1* gene locus have also been implicated in drug response. It has been reported that the SNP e27/T3421A (S1141T) (rs2229107) conferred decreased resistance to daunorubicin

in an *in vitro* setting, while the SNPs e5/C266T (M89T) (rs35810889), e17/T1985C (L662R) (rs35657960), and e17/C2005T (R669C) (rs35023033) all increased resistance to at least two of the drugs studied, which also included doxorubicin, valinomycin, and actinomycin D). In the same study e27/T3322C (W1108R) (rs35730308) reportedly decreased resistance to valinomycin when expressed in haplotype with e17/C2005T [208]. It should be noted, however, that opposing studies indicate that many of the SNPs do not influence P-gp function [209,210].

5.8.3 Effect of SNPs on the Function, Structure, and Expression of ABCC1 (MRP1)

SNPs at the *ABCC1* gene locus were found to be in low LD and high haplotype diversity when ~480 individuals from Chinese, Malay, Indian, European Caucasian, and African-American populations were screened [175]. Despite the large number of exonic SNPs identified in this gene, relatively few have been associated with drug response or disease progression. One SNP, e13/C1684T (rs35605), was reported to influence the pharmacokinetics of the irinotecan metabolites SN-38 and APC. However, it was highlighted that this may be due to alternative causative polymorphisms possibly in other membrane transporters [211]. Similarly, the SNPs e2/C218T (T73I) (rs41494447) and e23/G3173A (R1058Q) (rs41410450) were implicated in reduced resistance to at least one of the several drugs including daunorubicin, doxorubicin, etoposide, vinblastine, or vincristine in HEK293 and CHO-K1 cells. In the case of e17/G2168A (R723Q) (rs4148356), the SNP was found to confer reduced resistance to all drugs examined, while e2/G128C (C43S) (rs41395947) did not influence the efficacy of any agent [212]. Additional SNPs influencing the function of MRP1 include the SNP e28/G4009A (A1337T) (rs28364006) that was reported to influence the efficacy of methotrexate together with the intronic SNPs I22/G-1960A (rs2238476) and I9/T-176 C (rs35592) [213]. The e10/G1299T (rs60782127) polymorphism, which confers the amino acid exchange R443S, reportedly increased doxorubicin resistance while decreasing the transport of a number or organic anions [214]. Furthermore, the e2/G128C (C43S) (rs41395947) polymorphism, which was discovered not to influence protein expression or stability [215], was found to disrupt protein localization to the plasma membrane. Association between this SNP and reduced resistance to doxorubicin has also been highlighted [216]. In contrast to these positive associations, the results of a study that assessed the functional significance of 10 nonsynonymous SNPs in HEK293T cells reported that none altered the expression levels of MRP1 or have any impact on protein synthesis or stability [215].

5.8.4 Effect of SNPs on the Function, Structure and Expression of ABCG2

Although several SNPs have been identified in the *ABCG2* gene, few have been characterized and shown to influence the drug transport activity of the protein. One well-characterized nonsynonymous SNP, e5/C421A (Q141K) (rs2231142), is located between Walker A and signature motif C in the NBD. This SNP is associated with a less stable BCRP that is susceptible to proteosomal degradation and diminished localization to the plasma membrane [217]. This SNP was shown to reduce ATPase activity in HEK-293 cells, which was accompanied by a reduced mitoxantrone efflux [218]. An independent study also implicated e5/C421A (Q141K) (rs2231142) in drug

response after reporting an association between the heterozygous variant of the SNP and the onset of diarrhea following gefitinib treatment. Interestingly, however, there was no association in the same cohort with skin toxicity, another common side effect of gefitinib exposure [219]. The same SNP has been implicated in the pharmacokinetics of diflomotecan [220], imatinib [221], atorvastatin and rosuvastatin [222], and methotrexate [213]. SNP e5/C421A (Q141K) (rs2231142) was found not to affect the pharmacokinetics of the camptothecin analog, irinotecan [223] or the drug, lamivudine [200]. Two other nonsynonymous SNPs (V12M and Q126X) at the *ABCG2* gene locus also did not affect pharmacokinetics of the drug lamivudine [200].

5.8.5 Effect of SNPs in Other ABC Proteins

As previously mentioned, potentially functional SNPs have been reported in 12 ABC genes, with several of these associated with drug resistance or disease progression. A synonymous SNP, e15/C2127T (rs908832), found in the *ABCA2* gene was associated with early onset of Alzheimer's disease [224], while a nonsynonymous SNP in the *ABCB11* gene, e13/T1331C (rs2287622), was associated with altered canalicular expression of the protein leading to cholestasis [225,226]. Also, within the *ABCB11* gene, e28/G236A (rs473351), was found to be associated with intrahepatic cholestasis of pregnancy [227]. A few SNPs at the *ABCC2* gene locus have been associated with a defined phenotype including e10/G1249A (rs2273697), which was correlated to *ABCC2* mRNA expression in preterm babies but not in the human placenta [228], and e1/C−24 T (rs717620), which was associated with *ABCC2* mRNA levels in normal kidney cortex [229] as well as alterations to the pharmacokinetics of methotrexate in pediatric ALL patients [230]. Similarly, the SNP e10/C1446G was associated with alterations to hepatic expression of MRP2 [231], while the SNPs e18/C2366T (rs56220353) and e31/G4348A (rs56296335) were correlated to both expression level and membrane localization of MRP2 in a kidney cell line [232]. The SNP −5′UR/C211T, which resides in the *ABCC3* gene, was found to be associated with ABCC3 mRNA levels in human liver tissue [233], yet does not affect mRNA levels in ALL patients [234]. Similar associations were reported for two SNPs located in the *ABCC6* gene, with e24/C3421T (rs72653706) being correlated with a higher susceptibility to early cardiovascular disease [235] and the nonsynonymous SNP E27/G3803A (rs2238472) associated with plasma levels [236]. The final ABC gene to contain an SNP with reported associations to an established phenotypic property is *ABCC11* . SNP e5/G538A (rs17822931) at the *ABCC11* gene locus was found to be associated with earwax type [237].

5.8.6 Diseases Arising from ABC Gene Mutations

Several familial diseases are known to arise from loss-of-function mutations in ABC transporter genes (Table 5.3). Diseases have been associated with each subclass of ABC, with the exception of ABCE and ABCF, which are not associated with active substrate transport.

Familial high density lipoprotein (HDL) deficiency, a genetic disorder transmitted as a dominate trait, and the rarer autosomal recessive disease, but clinically similar, Tangier's disease are associated with defective ABCA1 [238]. These diseases result

TABLE 5.3 Diseases Associated with Mutations in Genes Encoding Members of the ABC Superfamily

Disease	Associated ABC Transporter	References
Tangier disease, high density lipoprotein deficiency	ABCA1	238,239
Stargardt disease and age-related macular degeneration	ABCA4	4,240
PFIC	ABCB11 (PFIC-II) ABCB4 (PFIC-III)	241,242
Immune deficiency	ABCB2, ABCB3	2
Sideroblastic anemia with ataxia	ABCB7	243
Dubin–Johnson syndrome	ABCC2	244
Pseudoxanthoma elasticum	ABCC6	245
Cystic fibrosis	ABCC7	246,247
PHHI	ABCC8, ABCC9	248
X-linked adrenoleukodystrophy	ABCD1	9
Sitosterolemia	ABCG5, ABCG8	249

Abbreviations: PFIC, progressive familial intrahepatic cholestasis; PHHI, persistent hypoglycemia in infants.
Source: Adapted from Ref. 2.

from loss-of-function mutations, of which, over 50 different mutations have been identified within the *ABCA1* gene [239]. Both diseases are characterized by a complete absence of HDL in the blood plasma and subsequently an increased incidence of cardiovascular disease [238,239]. Similarly, various macular disorders, such as Stargardt's disease and age-related macular dystrophy, are linked to mutations in the *ABCA4* gene. This gene encodes the ABCR protein, which is exclusively expressed in photoreceptors and involved in the transport of vitamin A derivatives into the retinal pigment epithelium (RPE) [4]. Degeneration of rods and cones is the ultimate result of expressing loss-of-function mutations in *ABCA4* [240].

As previously highlighted, SNP mutations in *ABCB11* have been associated with the onset of cholestasis; however, it is important to note that mutations in *ABCB4* also contribute to the disease in a similar fashion. Mutations in *ABCB4* and *ABCB11* have been found to cause progressive familial intrahepatic cholestasis (PFIC) types III and II, respectively [241]. Cholestasis, which is characterized by a retention of bile salts in the liver, an absence of biliary phospholipids and elevated bile acid levels in blood plasma ultimately leads to liver failure and, without transplant, patient death [241]. In each disease, mutations in the responsible proteins produce a dysfunctional protein or a phenotype completely lacking detectable protein. This was observed in a screen of 50 PFic3 patient samples, where ~30 different mutations were observed in the *ABCB4* gene. In this case, no ABCB4 protein was detected following immunostaining of the samples from liver failure patients and this lack of detectable protein was proposed to result from the presence of a premature stop codon and subsequent degradation of mRNA [242,250,251]. In other examples, missense mutations at the Walker A and Walker B motifs of the *ABCB4* gene locus were observed, which prevented ATP binding and hydrolysis [242,250].

One of the most prominent diseases associated with the ABC proteins is CF, which results from mutations in the gene known as *cystic fibrosis transmembrane regulator*

(*CFTR*) or *ABCC7*. Characterized by profuse mucosal secretions within the lungs and an increased vulnerability to bacterial infections, CF continues to be associated with an average life span in patients of just 34 years [246]. Although there have been over 1000 disease-associated mutations described in the *CTFR* gene, there are several that are consistently found in large proportions of CF patients [247]. The most common mutation, known as $\Delta F508$, is present in over 90% of CF patients in the United States and produces a protein lacking a phenylalanine residue at position 508 [247]. Three-dimensional modeling of the CTFR protein highlights the importance of this residue in facilitating an interaction in the tertiary structure between the surface of NBD1 and the cytoplasmic loop CS4 in the C-terminal TMD (TMD2) [252]. Despite the defective protein retaining its chloride channel function in cell-free *in vitro* studies, *in vivo* studies revealed that the ΔF508 mutation resulted in a misfolded protein that is swiftly degraded before it localizes to the plasma membrane [247].

From the ABCG family, it is has been established that deficiency in functional ABCG5 and ABCG8 proteins produces the autosomal recessively inherited disease, sitosterolemia. This is a rare disease with only 45 cases reported in the literature worldwide and produces significantly elevated levels of plant sterols in the blood plasma. While a healthy individual retains only 5% of the plant sterols consumed in a regular diet, sitosterolemia patients retain 10–25 times more, which ultimately produces coronary and aortic atherosclerosis leading to cardiac arrest [249].

5.9 CONCLUSIONS AND FUTURE PERSPECTIVES

MDR is a problem that diminishes the efficacy of chemotherapy in a range of diseases. It continues to hamper the development of new drugs and also prevents the optimization of new combinations of existing therapies. MDR may be intrinsic to some cell types or acquired following extended exposure to chemotherapy and most often results from a complex network of mechanisms that include drug metabolism, drug efflux, and alterations to the drug target. Despite often conflicting evidence on the role of the ABC transport proteins in clinical MDR, it is clear from decades of research that the ABC transport proteins contribute significantly to this phenomenon.

With the limited success in modulating or inhibiting the function of the ABC transport proteins, it can be argued that this family of proteins has failed as a therapeutic target. Although only meant as adjuvant therapies, the existing ABC transport protein modulators and inhibitors have proven ineffective in reversing MDR and continue to be associated with high toxicities at dose concentrations required to effectively inhibit the target protein. That said, to develop agents that improve existing chemotherapy regimens and diminish the effects of MDR, the ABC transport proteins must be targeted. Molecular mechanisms of inhibition such as RNA interference may prove more effective than small-molecule treatment. However, many ABC transporters are ubiquitously expressed and play an important physiological role. Hence, the specificity of any modulator or inhibitor must be improved in order to reduce the onset of adverse effects.

Attempts to develop novel strategies to counteract the effects of MDR will undoubtedly benefit from the recent interest in the pharmacogenomics of the ABC drug transporters. With growing evidence to suggest that interindividual differences in response to therapy are linked to the presence of SNPs in the coding and promoter regions of the

ABC genes and their protein regulators, it is clear that a better understanding in this field will permit chemotherapy protocols to be better tailored to individual populations.

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