ABC Efflux Pump-Based Resistance to Chemotherapy Drugs

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1. Introduction and Brief History

Over 1 million new cases of all types of cancer are diagnosed each year in the United States alone, and 559 650 cancer-related deaths were expected to occur in 2007.[1] Chemotherapeutic treatment of cancers is an important tool to combat this devastating disease. However, chemotherapy can fail due to the development of tumor cell resistance to multiple drugs, a phenomenon known as multidrug resistance (MDR), and essentially all cancer-related deaths are considered to be a result of chemotherapy failure.[2] MDR can have many causes, but one important mechanism of drug resistance is the expression of active drug efflux pumps in the membranes of cancer cells.

The efflux pump proteins mediating MDR in human cancers belong to the ATP-Binding Cassette (ABC) superfamily of proteins. This is a diverse group of (primarily) active membrane transporters and is one of the largest known protein families.[3] Its members include proteins that mediate a variety of transport processes, in both prokaryotes (e.g., membrane-embedded importers for sugars and amino acids, and drug exporters) and eukaryotes, where only ABC exporters are known. The 49 ABC proteins identified in the human genome[4] (there appear to be 48 functional proteins) are involved in the efflux of substrates such as phospholipids, sterols, bile salts, and amphipathic drugs. While as many as 15 ABC transporters have been observed to export chemotherapy drugs using in vitro experimental systems,[5] only 3 transporters have thus far been implicated as major contributors to MDR in cancer. Discovered over 30 years ago, P-glycoprotein (Pgp; MDR1; ABCB1), was the first mammalian ABC protein to be identified. Multidrug resistance-associated protein 1 (MRP1 or ABCC1) and more recently ABCG2 (also termed the breast cancer resistance protein, BCRP, or mitoxantrone resistance protein, MXR) also appear to function as clinically relevant drug efflux pumps. All three proteins were discovered as factors overexpressed in MDR cell lines in culture, and they have since been detected in MDR tumors in patients. Considerable effort has been made to understand the structure and function of these three drug pumps, which possess a unique ability to recognize and transport a broad array of structurally diverse compounds out of the cell. The development of compounds known as modulators, which are able to reverse MDR by blocking or inhibiting the ATP-dependent transport function of these proteins, is also an important goal of researchers in this field. This review will summarize our understanding of the substrate specificity of the mammalian drug efflux pumps, their structure, the molecular mechanisms by which they exert their function, and the progress made to date in reversing pump-mediated MDR.

2. Cellular Resistance to Chemotherapy Drugs

2.1. MDR in Cultured Cell Lines

For well over 30 years, it has been known that cultured cell lines subjected to increasing concentrations of various cytotoxic or chemotherapeutic drugs in vitro can develop resistance, which occurs by a variety of mechanisms.[6] The processes leading to drug resistance may include upregulation of cytochrome P450-based degradation of drugs; sequestra-

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tion of the compound from its target nucleic acid, enzyme, or organelle; increased DNA repair activity to counter drug-mediated damage; insensitivity to drug-induced apoptosis; and interference with the cellular entry and accumulation of the compound, such as by active removal of the drug from the cell.

Ling and co-workers first noted that Chinese hamster ovary cell lines selected for colchicine resistance displayed not only reduced permeability to colchicine but also resistance to a variety of other structurally unrelated compounds.[7] Alterations in energy-dependent membrane proteins appeared to be responsible for the MDR phenotype. In 1976, this group singled out a 170 kDa surface glycoprotein as the factor responsible for the altered cellular permeability to drugs, and named it P-glycoprotein.[8,9] A variety of MDR cell lines have since been developed by stepwise selection with colchicine, doxorubicin, and other drugs, and Pgp overexpression has been identified in many such cell lines from rodents, other mammals, and humans. Expression of Pgp was measured by quantitative PCR in 39 of 60 tumor cell lines used by the U.S. National Cancer Institute to identify new anticancer agents.[10] High levels of Pgp expression were found in renal and colon carcinomas, and all melanomas and central nervous system tumor cell lines also expressed the protein. Cole and co-workers selected the human small-cell lung cancer cell line NCI-H69 for resistance to doxorubicin, developing the H69AR multidrug resistant variant.[11] This cell line displayed cross-resistance to a variety of other cytotoxic compounds to which Pgp-overexpressing cell lines were also resistant, including colchicine, vincristine, and vinblastine. Curiously, however, no Pgp could be detected in the cells using Pgp-reactive monoclonal antibodies. The MDR cell line also had 6-fold lower glutathione (GSH) levels relative to the parent line, despite elevated GSH biosynthetic enzyme activity.[12] By generating cDNA clones from mRNA found in the drug-resistant cell line, Cole and co-workers identified a new ABC transporter gene whose expression was upregulated in the drug-resistant cell line.[13] This protein was first named MRP (multidrug resistance-associated protein) and later renamed MRP1 when other members of the ABCC subfamily were discovered. MRP1 expression has been detected in a large number of different tumor types, ranging from solid tumors such as gastrointestinal, breast, and kidney cancers to hematogical malignancies including acute myelogenous leukemia (AML) (reviewed in ref 14). High MRP1 expression levels are frequently noted in both small-cell and nonsmall-cell lung cancers.

It was later hypothesized that Pgp and MRP1 are not the only drug pumps involved in clinical MDR in cancer. Chen and co-workers[15] selected the human breast cancer cell line, MCF-7, for drug resistance by subjecting the cells to increasing concentrations of doxorubicin in the presence of verapamil, a compound known to inhibit Pgp function. The MCF-7/AdrVp progenitor cell line was 900-fold resistant to doxorubicin and also displayed resistance to some other drugs. However, it did not express Pgp, was no more resistant to the Pgp substrate vinblastine than the parent cell line, and had normal GSH levels. Doxorubicin resistance was found to be correlated with the overexpression of a 95 kDa membrane protein, whose level was reduced when the drug was removed from the medium but was not affected by the removal of verapamil. Depletion of ATP completely abrogated the efflux of rhodamine 123 (R123) and daunorubicin from these cells, indicating that an ATP-dependent efflux transporter was likely responsible for the drug-resistance phenotype.[16] By isolation of mRNA overexpressed in MCF-7/AdrVp cells, Doyle and co-workers identified a 655-residue ABC protein (a "half-transporter"), which they termed the breast cancer resistance protein (BCRP; later renamed ABCG2).[17] Two other research groups discovered the protein independently, naming it MXR[18] (mitoxantrone resistance protein) and ABCP[19] (placenta-specific ABC transporter).

Cell lines in culture have been invaluable models for the identification of literally hundreds of structurally diverse compounds to which drug pumps mediate resistance, as well as many modulators that reverse resistance to these compounds. However, the selection of cultured cells using drugs differs considerably from the emergence of MDR tumors in vivo, and the fact that drug efflux pumps are overexpressed and mediate resistance in vitro does not prove that these proteins are necessarily responsible for drug resistance in human cancers.

2.2. Clinical Drug Resistance in Human Tumors

During the course of chemotherapeutic drug treatment, some tumors are found to be unresponsive as a result of various adaptations. Cancers are often encountered that either are intrinsically drug-resistant or are initially drug-sensitive but later recur in a drug-resistant form. Some tumors develop resistance to a single therapeutic drug through a specific adaptation, and resistance can be easily circumvented through administration of an alternative drug therapy. However, cancer cells frequently display resistance to killing by multiple structurally diverse chemotherapeutic compounds (multidrug resistance, MDR). MDR is a major challenge to the successful treatment of many types of cancer, including breast, ovarian, colorectal, pancreatic, and kidney tumors. The latter three tumor types are frequently found to be intrinsically drug resistant,[20−23] likely because they are derived from tissue with innately high Pgp expression levels, whereas the former tumor types typically become resistant upon recurrence following one or more rounds of chemotherapy treatment.[24,25]

It has proved surprisingly difficult to demonstrate the importance of drug efflux pumps in MDR cancers. One reason for this is that multiple resistance mechanisms are commonly upregulated in tandem in many MDR cells. For example, Pgp is highly expressed in kidney cancer, yet these tumors are resistant to both Pgp drug substrates and drugs not within its spectrum, indicating that other resistance mechanisms must also be present (reviewed in ref 26). If there are redundant mechanisms for resistance to drugs that are Pgp substrates, inhibition of this transporter alone may not sensitize the cells. There has been more success in demonstrating a role for drug efflux pumps in cancers derived from tissues that typically do not express high levels of these proteins. The expression of Pgp in such tumor cells often correlates with a weak response to chemotherapeutic compounds and poor clinical prognosis for the patient.[27] For example, in treatment of ovarian cancer with paclitaxel, Pgp overexpression was reported to correlate inversely with probability of survival.[28]

In AML, flow cytometry has shown expression of Pgp in up to 50% of clinical samples. Levels of the protein were increased in tumors initially refractory to chemotherapeutic treatment and upon recurrence,[29] and expression correlated well with poor clinical prognosis. Others have shown that Pgp expression is more common in older patients, and younger AML and acute lymphoblastic lymphoma (ALL) patients typically show a better response to treatment.[30,31]

The discovery of ABCG2 in 1998[17] suggested that this protein might be responsible for some instances of MDR in tumors where Pgp was shown not to be the sole cause. However, a clear clinical role for ABCG2 in cancer treatment failure remains to be demonstrated. Using immunohistochemistry, Diestra and co-workers showed expression of ABCG2 in a wide variety of untreated solid tumor samples, including adenocarcinomas of the digestive tract, endometrium, and lung, and in melanomas.[32] However, the clinical relevance of this expression awaits further studies. The role of ABCG2 as a cause of MDR in AML and ALL cancers also continues to be debated.[31,33]

The role of MRP1 in MDR tumors is similarly controversial. Its frequency of expression in lung cancers has been estimated at 80−90%, and the protein has also been detected in solid tumors and leukemias, including some breast, prostate and ovarian cancers, gastrointestinal carcinoma, melanoma, neuroblastoma, chronic lymphocytic leukemia (CLL), and AML.[14,26] However, further work is required to define the exact functional significance of MRP1 expression in chemotherapeutic drug treatment.

Since the development of the Pgp substrate technetium-99m methoxyisobutylisonitrile (^{99m}Tc-MIBI), visualization of Pgp function in tumors in humans in vivo has been possible using scintigraphic studies. Reduced uptake and enhanced clearance of ^{99m}Tc-MIBI in sarcoma, lymphoma, lung cancer, and breast cancer has been observed to correlate with poor prognosis in patients.[34,35] However, ^{99m}Tc-MIBI is a substrate for MRP1 as well,[26] and the high expression levels of this drug pump in lung tumors could be responsible for the observed ^{99m}Tc-MIBI response.

3. Physiological Role of Drug Efflux Pumps

3.1. Tissue Distribution and Protective Function of Pgp

Pgp is present at low levels in many tissue types in humans[36] and rodents (which express two closely related Pgp proteins[37]) but is generally expressed at higher levels in epithelial cell surfaces throughout the body. It is found exclusively at the apical surface of cells in the kidney proximal tubule, canalicular membrane of hepatocytes, pancreas, the villous membrane of the small and large intestine, and the adrenal gland.[36,37] Pgp is also located in blood−tissue barriers, including the placenta and endometrium, blood−inner ear barrier, blood−mammary tissue barrier, blood−testis barrier, blood−nerve barrier, and endothelial cells of the blood−brain barrier, where it is exclusively oriented to transport substrates toward the blood.[38−45] It is reasonable to assume that Pgp has a major role in these locations in either restricting drug entry to the body via the gastrointestinal tract and excreting metabolites into the urine or gastrointestinal tract, or in preventing their access from the blood to the fetus and sensitive organs such as the brain and testis.

In fact, knockout mice lacking one or both drug-transporting Pgp proteins appear normal in every way, are fertile, and have a normal life span. However, when challenged with amphipathic drug substrates, these compounds accumulate in the brain to a much greater extent than in wild-type mice, resulting in neurotoxicity.[46] For example, knockout mice are 100-fold more sensitive to the toxicity of the pesticide ivermectin, which is a Pgp substrate. Experiments using radiolabeled Pgp substrates including ^{99m}Tc-MIBI have demonstrated that Pgp-mediated drug transport occurs in vivo in humans, not only in MDR tumors but also in normal tissues such as the blood−brain barrier, and that this transport is inhibited by known Pgp modulators.[35,47,48] The presence of Pgp in hematopoietic progenitor cells of the bone marrow protects these vital cells from toxic drugs during chemotherapy.[49] Thus, one important role of Pgp in the body is likely protection of tissues from toxic compounds. Since it is expressed in the intestinal epithelia, Pgp has a major impact on the bioavailability of orally administered drugs and the targeting of drug treatments to tissues such as the brain. Collie dogs often have a high sensitivity to invermectin due to a frameshift mutation in Pgp, which renders it nonfunctional in animals of one lineage.[50] However, a similar lack of functional Pgp has never been reported in humans, despite the common use of drugs that are Pgp substrates in the treatment of many diseases.

3.2. Other Potential in vivo Functions of Pgp

In addition to its protective role, the in vivo functions of Pgp may also include the transport of endogenous molecules or metabolites. While Pgp can act as a phospholipid flippase (see section 7.2), it is unlikely that that this is its primary function in vivo because the rate of flipping is relatively low,[51] and Pgp was unable to rescue a knockout of the liver phospholipid flippase (ABCB4), despite the fact that both proteins were expressed in the canalicular membrane.[52] However, it is possible that differing expression levels of each protein could account for this observation. Reconstituted Pgp has been shown to be an outwardly directed flippase for fluorescent phosphatidylethanolamine (PE) and phosphatidylserine (PS) derivatives.[51] The plasma membrane aminophospholipid translocase normally maintains PE and PS on the inner leaflet.[53] Thus, if the role of Pgp were to flip these natural lipids from the inner to the outer leaflet to a significant extent, it would counteract the action of the translocase, resulting in a futile cycle of phospholipid flip-flop accompanied by ATP hydrolysis. Pgp is also able to translocate fluorescent derivatives of simple glycosphingolipids such as glucosylceramide (GlcCer).[54,55] Pgp localized in the Golgi apparatus may thus have a role in the flipping of GlcCer from the cytoplasmic leaflet to the luminal leaflet, which is required during the biosynthesis of more complex glycosphingolipids.[55] Other likely endogenous Pgp substrates include β-amyloid peptides,[56] interleukins,[57] and steroid hormones such as aldosterone[58] and β-estradiol-17β-D-glucuronide.[59] Indeed, the transporter is known to interact with molecules such as progesterone and a variety of peptides.[60] A role in hormone/cytokine transport could explain Pgp expression in tissues such as the adrenal gland, hematopoietic cells, and lymphocytes.

3.3. Physiological Role of ABCG2

mRNA analysis indicates that ABCG2 is most highly expressed in the placenta, with high levels also found in the brain, liver, prostate, and small and large intestine.[17] Like Pgp, ABCG2 is also localized to the apical face of polarized membranes and is found in epithelial cells of the intestine, human placenta syncytiotrophoblast, liver bile canaliculi, lobules and lactiferous ducts of the mammary gland, and renal tubules, as well as the endothelium of veins and capillaries, including those at the blood−brain barrier and the placenta.[17,61−63] ABCG2 expression increases greatly in the mammary gland during lactation, where it transports chemotherapeutic drugs into the milk of humans, cows, and mice.[64−66] This may be a consequence of its apparent physiological role during lactation of secreting riboflavin (vitamin B_2) into milk.[67]

However, unlike the case of Pgp, ABCG2 knockout mice show phenotypic changes in the absence of the administration of a drug substrate. Such mice exhibit a much higher concentration of unconjugated bilirubin in blood plasma and the heme precursor protoporphyrin IX in both plasma and erythrocytes.[68] Additionally, they are 100-fold more sensitive to pheophorbide a, a phototoxic porphyrin catabolite of chlorophyll that accumulates and causes lethal phototoxic lesions when mice are fed alfalfa leaf concentrate.[68] This observation suggests that ABCG2 has a role in limiting absorption of heme and chlorophyll catabolites in the intestinal lumen. In certain stem cells, ABCG2 appears to prevent accumulation of porphyrins, which improves cell survival during hypoxia.[69] Inhibiting ABCG2 function reduces this survival advantage, and lowering heme biosynthesis restores it, indicating a direct interaction of ABCG2 with porphyrins.

3.4. Physiological Role of MRP1

MRP1 is expressed at low levels throughout many normal tissues and cell types in the body,[70] but it is more highly expressed in the adrenal gland, bladder, choroid plexus, colon, erythrocytes, kidney, lung, placenta, spleen, stomach, testis, helper T-cells, and muscle (both skeletal and cardiac).[14,70−74] In contrast to Pgp and ABCG2, MRP1 is localized to basolateral membranes in polarized cells.[75] MRP1 knockout mice are viable and fertile; however, drug sensitivity in some tissues that normally express high levels of MRP1, such as the kidney, testis, and bone marrow, is increased significantly.[76,77] The basolateral localization of MRP1 serves to protect sensitive tissues. For example, basolateral expression of MRP1 in Sertoli cells of the testis protects germline cells of the testicular tubules from toxic xenobiotics in the blood by outward efflux. Likewise, basolateral expression of MRP1 in the choroid plexus allows the protein to transport drugs from the cerebrospinal fluid to the blood to protect sensitive nervous system tissues.[78]

An additional phenotypic change in MRP1 knockout mice is a decreased response to inflammatory stimuli.[76] Leukotriene C_4 (LTC_4) is an inflammatory mediator synthesized in a variety of locations, including eosinophils and mast cells. Its secretion in response to IgE-mediated inflammation is reduced in MRP1 knockout mice.[76] LTC_4 is a very high affinity substrate for MRP1, and its transport by the protein, together with reduced glutathione, has been demonstrated experimentally.[79,80] MRP1 appears to export its substrates either in the form of glutathione conjugates or with cotransported reduced glutathione (GSH).[81] GSH alone is a poor MRP1 substrate, but GSH in the presence of hydrophobic substrates is transported much more readily, as is oxidized glutathione (GSSG).[82] Thus, MRP1 likely plays an important role in glutathione homeostasis in the body. Finally, like ABCG2, MRP1 may have a role in the protection of cells from neurotoxic bilirubin, as both glucuronide-conjugated and unconjugated bilirubin are transported by the protein.[83−85]

4. Structure of ABC Superfamily Drug Efflux Pumps

4.1. Domain Structure of ABC Proteins

Pgp, MRP1, and ABCG2 are members of the large ABC protein superfamily,[3,4] whose members likely evolved from a single ancestral membrane transporter gene. Thus, all ABC proteins share similarities in domain organization. The prototypical ABC transporter consists of four domains. Two of these domains are membrane-embedded (the transmembrane domains, TMDs), and each typically consists of 5−10 membrane-spanning α-helices, which contain the substrate-binding sites and/or pathway through which substrates are transported. The other two domains are the cytoplasmically localized nucleotide-binding domains (NBDs), which are intimately associated with each other, as well as with the membrane-embedded domains. The highly conserved NBDs contain a Walker A and Walker B motif, commonly found in nucleotide-binding proteins, and a signature C motif (LSGGQ) that is characteristic of the ABC superfamily. The

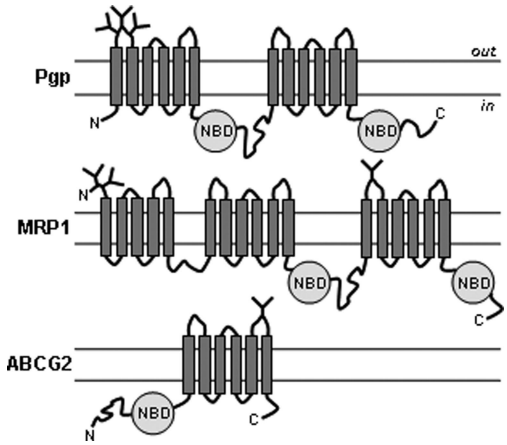

Figure 1. Topological models of the three ABC multidrug efflux pumps involved in resistance to chemotherapy drugs. Pgp and MRP1 are single polypeptides, while ABCG2 is a half-transporter that functions as a homodimer. Pgp comprises two homologous halves arising from a gene duplication, each with 6 TM helices and a cytoplasmic NBD. MRP1 displays a similar topology to Pgp but possesses an extra N-terminal domain of unknown function with 5 TM helices and an extracellular N-terminus. In contrast to the other proteins, the NBD of ABCG2 is located at the N-terminal end of the protein, and the 6 TM helices are located at the C-terminal end. N-Linked glycosylation is present on extracellular loops and turns of all three proteins, as well as at the N-terminus of the first TM domain of MRP1. Drug transport by all three proteins is powered by ATP hydrolysis at their cytosolic NBDs.

functional role played by each of these three motifs has been examined using mutational analysis.[86] The NBDs bind and hydrolyze ATP, thereby inducing conformational changes in the coupled TMDs that power the substrate-transport process.

In prokaryotes, the core domains of ABC transporters may be expressed as individual polypeptides, or they may exist in a variety of fused-domain polypeptide organizations.[87] However, in mammalian systems, ABC transporters exist exclusively in one of two forms, either as full transporters (comprising all four domains), or half-transporters (comprising a single TMD and NBD) that assemble to form homodimers or heterodimers. ABCG2 is a 655-residue half-transporter that possesses an N-terminal NBD and a 6-helix TMD (Figure 1). The functional protein is assumed to operate as a homodimer. Pgp is thought to have arisen from an internal duplication of an ancestral gene and exists as a full transporter with the typical topology and subunit architecture for a fused polypeptide ABC protein. The protein possesses cytosolic N- and C-termini, two membrane spanning domains of 6 helices each, and two NBDs in a single 1280-residue polypeptide (Figure 1). However, unlike ABCG2, the NBDs of Pgp are C-terminal to the TMDs. The structure of the 1531-residue MRP1 (Figure 1) is similar to that of Pgp, but the protein possesses an extra N-terminal TMD with 5 transmembrane (TM) helices, termed TMD_0, whose function remains unclear. TMD_0 is not required for substrate transport or membrane trafficking of normal MRP1;[88] however, the protein exists as a dimer in the membrane, and TMD_0 and the linker region may be required for dimerization.[89]

4.2. Structure of Entire Bacterial ABC Proteins

The sequence of the NBDs of all ABC proteins is highly conserved, and substantial high−resolution structural information on these domains has become available over the past 10 years. The first X-ray crystal structure of an ABC protein

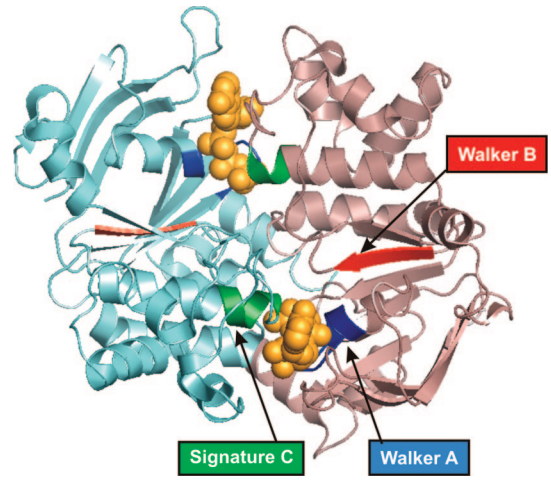

Figure 2. High-resolution X-ray crystal structure of the nucleotide sandwich dimer of the catalytically inactive H662A mutant of HlyB,[322] the ATP-binding subunit of the hemolysin exporter of *E. coli* (pdb 1XEF). The Walker A motif (blue), the Walker B motif (yellow), and the signature C motif (red) are shown in both subunits, and the two bound ATP molecules are displayed in space-filling representation (orange).

domain to be solved (to 1.5 Å) was that of HisP, the soluble NBD subunit of the bacterial histidine permease, complexed to ATP in the absence of Mg^{2+}.[90] The HisP structure displayed a characteristic L-shaped monomer that has since been found in all NBDs of ABC proteins. Subsequent structures that were solved for several NBD dimers and whole ABC proteins revealed an interdigitated "69"-type head-to-tail arrangement of the subunits, in which the binding sites for two molecules of ATP are created at the subunit interface (see Figure 2). A so-called ATP sandwich dimer is created by the Walker A and Walker B motifs of one NBD and the signature C (LSGGQ) motif of the opposing NBD.[91−96]

It should be noted that these stable dimeric structures with two bound nucleotides were only observed when the NBDs were catalytically inactivated by mutation of a critical residue, or when Mg^{2+}, an essential cofactor, was absent. The bacterial vitamin B_{12} transporter BtuCD was the first complete ABC protein structure to be solved (at 3.2 Å resolution) and shows two closely associated cytosolic NBDs, along with a bundle of TM helices lining the translocation pathway, which was closed by a cytoplasmic gate.[92] However, BtuCD is an importer with 20 TM helices, and this protein (and other bacterial ABC importer structures solved recently[94,97,98]) may not be good structural models for mammalian ABC drug exporters.

Recently, the structure of the *Staphylococcus aureus* ABC protein Sav1866 was solved at 3.0 Å resolution bound to ADP[93] (Figure 3A) and at 3.4 Å resolution bound to the nonhydrolyzable ATP analogue, adenosine 5′-($\beta\gamma$-imido)-triphosphate (AMP-PNP).[99] Sav1866 is a homodimeric half-transporter that resembles the N-terminal half of Pgp in subunit organization, with the TMD lying N-terminal to the NBD (Figure 1). Sav1866 displays drug-activated ATPase activity[93] and has recently been shown to function as a multidrug export pump,[100] in keeping with the fact that its sequence shows 69% similarity and 30% identity to the N-terminal half of Pgp. The dimer possesses two closely interacting NBDs with bound nucleotide and TMDs comprising 12 TM helices that form a central cavity. In the Sav1866 structure, the central cavity is exposed to the outer

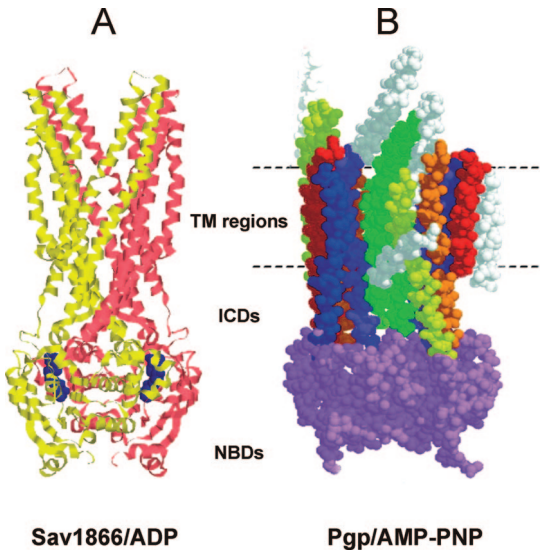

Figure 3. Structures of ABC multidrug efflux pumps. (A) High-resolution X-ray crystal structure in ribbon representation of the bacterial multidrug efflux pump Sav1866[99] from *S. aureus* bound to ADP (pdb 2HYD). The protein is a half-transporter that is assumed to function as a homodimer; the two monomers are displayed in yellow and pink, and the two molecules of ADP bound to the NBDs are shown in blue. (B) Medium resolution cryo-electron microscopic space-filling model of the structure of mammalian Pgp bound to the nonhydrolyzable nucleotide analogue, AMP-PNP (reprinted from ref 109 with permission from the American Chemical Society). The NBDs are shown in purple (the bound nucleotide molecules are not visible). Eight of the TM helices are colored in pairs to indicate the symmetry between the two halves of the protein; the four helices shown in gray do not show an obvious symmetry relationship. The presumed location of a 4.5 nm thick lipid bilayer is shown by the dotted lines. The locations of the TM regions, the ICDs, and the closely associated NBDs are shown for both transporters.

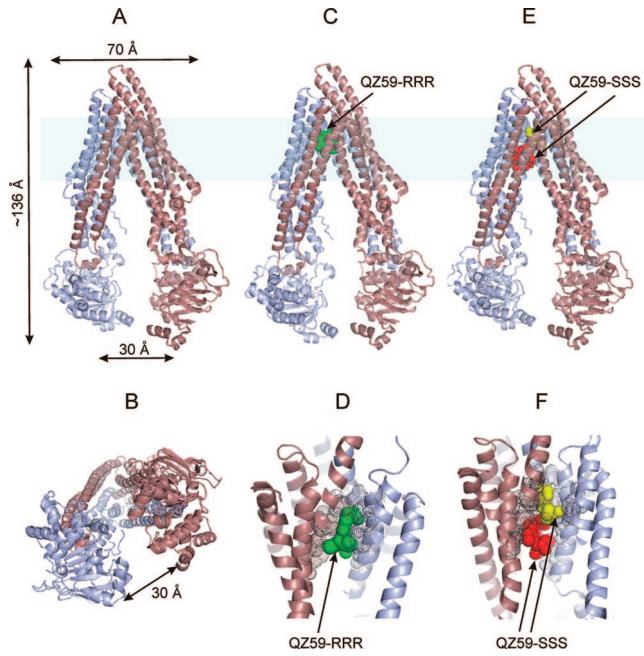

Figure 4. High-resolution X-ray crystal structures of Pgp. (A) 3.8 Å resolution structure of apo-Pgp (pdb 3G5U) shown from the side view, with the approximate location of the membrane indicated by the blue bar. The N-terminal portion of the protein is shown in pink, and the C-terminal portion is shown in blue. The approximate molecular dimensions of Pgp are indicated. (B) View of the NBDs shown from the cytosol looking up toward the membrane. (C) Structure of Pgp with a single molecule of QZ59-RRR (green) bound to the drug-binding pocket within the inner leaflet region of the membrane (pdb 3G60). (D) Close-up view of QZ59-RRR occupying the middle site in the drug-binding pocket with the volumes of nearby side chains shown in gray shading. (E) Structure of Pgp with two QZ59-SSS molecules (yellow and red) bound to the drug-binding pocket within the inner leaflet region of the membrane (pdb 3G61). (F) Close-up view of the QZ59-SSS molecules occupying the upper and lower sites in the drug-binding pocket with the volumes of nearby side chains shown in gray shading.

leaflet and extracellular space and is closed at the inner leaflet of the membrane, suggesting that the transporter is in an "outward-facing" conformation. This model implies that nucleotide binding/hydrolysis at the NBDs results in conformational changes at the TM domains, which switch the protein from an inward-facing to an outward-facing structure, thus effecting transport of its substrates.[101] The Sav1866 structure agrees well with that of the recently published structure (3.7 Å resolution) of the AMP-PNP-bound form of MsbA, a bacterial lipid A flippase.[95]

4.3. Structure of Pgp, MRP1, and ABCG2

At the time of writing, limited high-resolution structural information on intact mammalian ABC proteins has been published. Pgp is the transporter for which the most structural information is available. The proposed topology of Pgp (Figure 1) was first confirmed through the use of Cys mutagenesis[102] and epitope insertion with immunofluorescence.[103] Clarke and co-workers cross-linked the Walker A motif of one NBD of Pgp with the signature C motif of the opposing NBD,[104] confirming that these regions of the protein are in close proximity in Pgp, as they are in bacterial ABC proteins. Several low-to-medium resolution electron microscopic images have been reported for Pgp,[105−109] the best of which is a ~8 Å cryo-electron microscopy structure with bound AMP-PNP[109] (Figure 3B). This structure confirms the presence of two closely associated NBDs and TMDs consisting of 12 helices in total, which reorient upon ATP binding. The connectivity of the membrane helices could not be determined from the highest-resolution structure (Figure 3B), but cross-linking experiments have shown that TM helix 6 is close to TM10, 11, and 12, and that TM helix 12 is close to TM4, 5, and 6.[110] From Cys mutagenesis studies, the drug-binding sites of Pgp appear to reside in the membrane-embedded region, at the interface between the two halves of the protein, in TM helices 4−6 and 9−12.[111−115] Fluorescence studies have confirmed that the NBDs of Pgp are closely associated[116] and lie close to the membrane surface,[117] and showed that the drug-binding sites reside in the region of the protein located in the inner leaflet of the membrane.[118,119] A recent study used cysteine mutagenesis and chemical cross-linking to show that Pgp shares important features of its domain architecture with Sav1866; in particular, the long intracellular loops of one TMD appeared to contact the opposing NBD, a feature not observed in bacterial ABC importers.[120]

An important development in our understanding of mammalian ABC drug pumps was the recent publication of the 3.8 Å crystal structure of mouse Pgp in the absence of nucleotide[121] (Figure 4 A and B). The most remarkable feature of this "apo" structure is how well it agrees with both the bacterial ABC protein structures and the biochemical/biophysical data generated on Pgp structure and function over the past 30 years. The protein possesses a 2-fold axis of pseudosymmetry wherein each of two bundles of TM

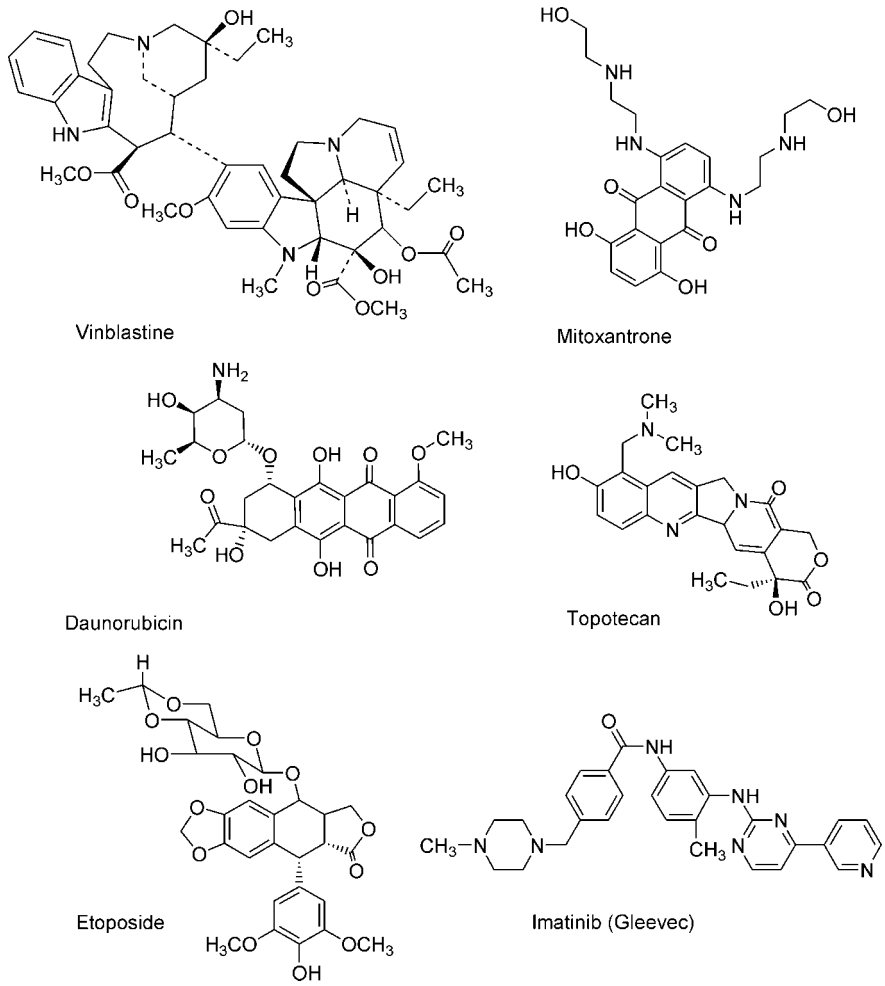

Figure 5. Structures of some chemotherapeutic drugs that are substrates for Pgp, MRP1, or ABCG2.

helices are composed of portions from both the N-terminal (Figure 4 A and B, pink) and C-terminal (Figure 4 parts A and B, blue) halves of the protein, bounding a 6000 Å^3 cavity within the lipid bilayer. This crossover is very reminiscent of the Sav1866 (Figure 3A) and corrected MsbA structures. The Pgp structure was solved in the absence of nucleotide, and the two NBDs are located ~30 Å apart (Figure 4A and B). The open-apo structure of MsbA from *E. coli* displays a much wider separation of the NBD domains. In contrast, one apo structure and the nucleotide-bound MsbA structures, as well as the structures of Sav1866 (both nucleotide-bound[93,99]) and other bacterial ABC proteins,[101,122] show a tight association of the NBDs. The arrangement of the NBDs is already controversial, since some evidence supports the wide apo-MsbA structure.[123−125] It remains unclear whether such an open structure exists for native MsbA, since it would require a dramatic conformational change to close the NBDs upon nucleotide binding/hydrolysis. While the NBD separation is only 30 Å in the Pgp structure, versus ~50 Å in MsbA, Aller et al. suggest that Pgp may open even wider to accommodate very large substrates.[121] Further work will be required to distinguish whether this open conformation is a real feature of native Pgp or a crystal-packing artifact.

The structure of the separately expressed N-terminal NBD of MRP1 with bound ATP and Mg^{2+} has been solved to 1.5 Å, revealing an NBD fold similar to that of prokaryotic ABC proteins.[126] While no high-resolution data on intact MRP1 and ABCG2 currently exist, electron microscopy has also been used to probe the structures of MRP1 to ~22 Å resolution[127] and ABCG2 to ~18 Å resolution.[128] The MRP1 structure showed monomers possessing a putative central pore of dimensions ~80 × 100 Å that interacted to form dimers, while the observed ABCG2 structures were proposed to be tetramers of ABCG2 dimers. Unfortunately, these low-resolution structures have contributed little to our understanding of the structure and function of mammalian multidrug transporters. Computational methods have been used to create 3-dimensional structural models for Pgp, MRP1, and ABCG2, based on the structures of bacterial ABC proteins, including Sav1866 and others.[129−132] It is anticipated that the Pgp crystal structure will provide a better starting point for modeling of ABCG2 and MRP1.

5. Substrate Specificity of ABC Drug Efflux Pumps

5.1. MDR Spectrum Substrates

Pgp, MRP1, and ABCG2 all exhibit unusual substrate promiscuity or "polyspecificity". Each protein has the potential to interact with literally hundreds of structurally diverse substrates (as exemplified in Figure 5), and the substrate spectrum of each, while not identical, overlaps to some extent (Table 1). An MDR substrate is classically defined as a molecule to which cells expressing the drug efflux pump exhibit resistance in cytotoxicity assays. At the molecular level, a substrate may also be considered as a small

Table 1. Chemotherapeutic Drugs and Other Compounds That Interact with ABC Multidrug Efflux Pumps

Pgp	MRP1	ABCG2
analgesics		
morphine		
antiarrhythmics		
amiodarone		
propafenone		
quinidine		
antibiotics		
erythromycin	difloxacin	ciprofloxacin
gramicidin D	grepafloxicin	norfloxacin
anticancer drugs		
anthracenes		
bisantrene		mitoxantrone
mitoxantrone		
anthracyclines		
doxorubicin	doxorubicin	
daunorubicin	daunorubicin	
camptothecins		
topotecan	topotecan	topotecan
	irinotecan	irinotecan
epipodophyllotoxins		
etoposide	etoposide	etoposide
teniposide	teniposide	teniposide
taxanes		
paclitaxel		
docetaxel		
Vinca alkaloids		
vinblastine	vinblastine	
vincristine	vincristine	
others		
	methotrexate	methotrexate
		flavopiridol
antiemetics		
ondansetron		
antiepileptics		
felbamate		
topiramate		
antihelminthics		
ivermectin		
antihistamines		
fexofenadine		
terfenadine		
antihypertensives		
reserpine		reserpine
propanolol		
antiviral drugs		
nelfinavir	ritonavir	lamivudine
ritonavir	saquinavir	zidovudine
saquinavir		
calcium-channel blockers		
azidopine		
diltiazem		
nifedipine		
verapamil		
calmodulin antagonists		
chlorpromazine		
trans-flupentixol		
trifluoperazine		
cardiac glycosides		
digoxin		
flavonoids		
		genestein
		quercetin
fluorescent dyes		
calcein-AM	BCECF	BODIPY-prazosin
H33342	calcein	H33342
rhodamine 123	fluo-3	
tetramethylrosamine		
folates		
	folic acid	
	L-leucovorin	
glucuronide conjugates		
	estradiol-17-β-D-glucuronide	estradiol-17-β-D-glucuronide
	etoposide glucuronide	
	glucuronosylbilirubin	
	NS-38-glucuronide	

Table 1. (Continued)

Pgp	MRP1	ABCG2
glutathione conjugates		
	aflatoxin B_1-epoxide-SG	dinitrophenyl-S-glutathione
	cyclophosphamide-SG	
	doxorubicin-SG	
	hydroxynonenal-SG	
	leukotrienes C_4, D_4, E_4	
	melphalan-SG	
	prostaglandin A_2-SG	
H_2-receptor antagonists		
cimetidine		
HMG-CoA reductase inhibitors		
lovastatin		cerivastatin
simvastatin		pravastatin
		rosuvastatin
immunosuppressive agents		
cyclosporin A		
tacrolimus (FK506)		
metalloids		
	potassium antimonite	
	sodium arsenate	
	sodium arsenite	
natural products		
colchicine		
curcuminoids	curcuminoids	curcuminoids
peptides		
N-acetyl-LLY-amide (ALLN)	reduced glutathione GSSG	
leupeptin	glutathione GSH	
pepstatin A		
valinomycin		
pesticides		
cypermethrin	fenitrothion	
endosulfan	methoxychlor	
fenvalerate		
methylparathion		
porphyrins		
		hematoporphyrin
		pheophorbide a
		protoporphyrin IX
steroids		
aldosterone		
corticosterone		
cortisol		
dexamethasone		
sulfate conjugates		
	dehydroepiandrosterone-3-sulfate	acetaminophen sulfate
	estrone-3-sulfate	dehydroepiandrosterone-3-sulfate
	sulfatolithocholyl taurine	estrone-3-sulfate
toxins/carcinogens		
	aflatoxin B_1	aflatoxin B
		PhiP
tyrosine kinase inhibitors		
gefitinib	gefitinib	gefitinib
imatinib mesylate	imatinib mesylate	imatinib mesylate
antialcoholism drugs		
disulfiram		

molecule that interacts specifically with the drug pump's binding pocket and is transported by the protein.

Substrates that bind to Pgp are generally large (200−1900 Da) organic molecules, amphipathic and lipid-soluble in nature, and they frequently possess aromatic ring systems. While some Pgp substrates are uncharged, many possess a positively charged N atom at physiological pH (reviewed in refs 5, 133, and 134). However, it is difficult to make generalizations about the properties of compounds that interact with Pgp, and many substrates have been identified that do not strictly conform to these descriptors. For example, a variety of linear and cyclic peptides and ionophores are known to interact with the protein,[135−137] yet peptides are smaller than typical substrates and often lack aromatic rings. Pgp substrates include classical chemotherapeutic drugs (such as anthracyclines, *Vinca* alkaloids, and taxols), new classes of anticancer agents such as tyrosine kinase inhibitors, human immunodeficiency virus (HIV) protease inhibitors, immunosuppressants, ionophores, peptides, fluorescent dyes, steroids, cardiac glycosides, and many others (Figure 5 and Table 1).

The search for specific structural characteristics common to all Pgp substrates has met with limited success. There is no common "pharmacophore" that can be used to identify a particular drug as a Pgp substrate. Seelig and co-workers examined over 100 compounds known to interact with Pgp and classified them based on the number and separation distance of electron donor groups.[138,139] All substrates examined were found to possess either 2 or 3 electron donor groups separated by 2.5 or 4.6 Å. Other researchers

subsequently suggested combinations of electron donors, hydrophobic groups, and/or aromatic rings in specific spatial organizations.[140−142] A more recent 3-dimensional approach suggested that molecules with two H-bond acceptors 11.5 Å apart and two H-bond donors 16.5 Å apart would be Pgp substrates.[143] If one of these structural classifications holds for all substrates, it will give an indication of how Pgp recognizes and binds its substrates in the drug-binding pocket and would allow us to predict a priori if new drugs are substrates for the transporter.

MRP1, unlike Pgp, transports most of its substrates (see Table 1) either as glutathione conjugates or together with free glutathione. The protein appears to contain two binding regions, one for amphipathic molecules with properties similar to Pgp substrates and a second more hydrophilic site for free glutathione or the glutathione portion of drug conjugates. MRP1 transports a wide variety of endogenous molecules, such as LTC_4, prostaglandins, glucuronide conjugates of steroids and bilirubin, sulfate conjugates, and toxins like aflatoxin B_1.[144,145] It also confers resistance to several anticancer drugs, including anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, and methotrexate (also an ABCG2 substrate), which is not transported by Pgp (Figure 5 and Table 1). MRP1 can also transport and confer resistance to anionic metalloids, including arsenate, arsenite, and antimonite.

Like Pgp and MRP1, ABCG2 is a polyspecific drug transporter, and although it shares much of its substrate complement with these proteins, there are some important differences (Table 1). Like MRP1 (but unlike Pgp), ABCG2 appears to transport both positively and negatively charged drugs, including sulfate[146,147] and glucuronide[148] conjugates. ABCG2 cannot transport anthracyclines or *Vinca* alkaloids (both substrates of Pgp and MRP1) or taxols and verapamil, which are Pgp substrates. However, it can transport epipodophyllotoxins such as etoposide and camptothecins such as topotecan, which are also substrates of both Pgp and MRP1. The new tyrosine kinase inhibitors imatinib (Gleevec) and gefitinib (Iressa) are substrates of all three efflux pumps; however, ABCG2 interacts with these drugs with much higher affinity. Thus, there seems to be some redundancy in substrate specificity between these three drug transporters.

5.2. Binding and Transport of Drugs

Substrates must bind to the drug-binding pocket of efflux pumps before the transport process can occur, powered by ATP binding/hydrolysis at the NBDs of the protein. Binding occurs with variable affinity, which is characteristic of each drug. Drug binding to Pgp has been characterized using a variety of approaches,[149] including photoaffinity labeling with reactive substrate analogues,[150,151] and by the effects of drugs on ATPase activity and transport rates. A few studies have employed plasma membrane vesicles containing Pgp and radioactive drugs in direct measurements of binding affinities.[152−156] Fluorescence-quenching techniques have also been used successfully, employing both Pgp labeled with a fluorescent probe and the intrinsic Trp fluorescence of the protein, to quantitate the equilibrium binding affinity of over 100 compounds.[60,157,158] Dissociation constants were found to cover almost 4 orders of magnitude; for example, colchicine binds to Pgp with relatively low affinity ($K_d \approx 150\ \mu M$),[60] while PSC-833 binds with very high affinity ($K_d \approx 25$ nM).[157] Drug binding to ABCG2 has typically been measured using radioactive substrates such as [^{3}H]daunorubicin or by employing photoaffinity labeling and its inhibition by other drugs.[159−161] A large number of studies have used photoaffinity labeling to estimate the affinity of drug binding to MRP1.[162]

Pgp-mediated drug transport has been determined by measuring basal-to-apical drug movement in polarized epithelial cells expressing the protein (for example, see ref 163). Inside-out membrane vesicles from Pgp-expressing cells, and reconstituted proteoliposomes containing purified Pgp, have also been employed in transport measurements with radiolabeled substrates, such as [^{3}H]colchicine,[164,165] [^{3}H]vinblastine,[164] and [^{125}I]-labeled peptides,[137] using rapid filtration techniques. Inhibition of drug transport by a second compound was taken as an indication that the compound bound to the protein and, thus, competed with the radiolabeled substrate.

More recently, real-time drug transport assays have been developed using fluorescent Pgp substrates such as tetramethylrosamine (TMR),[166,167] Hoechst 33342 (H33342),[167,168] and others,[169] thus allowing measurement of initial rates of transport. In reconstituted systems, Pgp can build up a substrate concentration gradient across the membrane of 5−6-fold for some substrates,[137,165] and as much as an 18-fold gradient for colchicine in plasma membrane vesicles,[164] and this gradient is collapsed by the addition of Pgp inhibitors.[166]

MRP1 transport has been well-characterized in membrane vesicles, where [^{3}H]LTC_4, [^{3}H]vincristine and other substrates,[81,170] and both GSH and GSSG are transported, although the K_m for transport of GSSG is considerably lower.[82] Carboxyfluorescein and a variety of other dyes have also been characterized as MRP1 substrates in fluorescence-based assays.[171−174] Transport in ABCG2 has primarily been characterized using fluorescent[175−177] or radioactive substrates[178] in intact MDR cells in culture, or cells transfected with the gene. More recent studies used membrane vesicles containing ABCG2 to characterize the transport process[147] and verified the partial overlap in substrates between ABCG2 and the other drug efflux pumps.

5.3. Multidrug-Binding Pockets

While drug binding is known to occur within the TMD of Pgp, and our understanding of where and how substrates bind to this protein is improving, the structure and exact location of the substrate binding site(s) in MRP1 and ABCG2 remain ill-defined. Using ATPase inhibition as a measure, Borgnia et al.[179] suggested a single binding site in Pgp for all substrates. Shapiro and Ling[180] proposed the existence of at least two interacting binding sites that display positive cooperativity in drug transport. One site was suggested to bind R123, other rhodamine drugs, and anthracyclines (the R-site), and a second site was proposed to bind H33342 and colchicine (the H-site).[180] Later, they identified a third site in Pgp that binds prazosin and progesterone.[181] Callaghan and co-workers[154] suggested the existence of multiple drug-binding sites that interact allosterically, based on measurements of radiolabeled drug binding to Pgp.

Current models of Pgp drug binding suggest that, rather than one or a few discrete drug-binding sites, there is a large, flexible drug-binding region, which the high-resolution crystal structure appears to confirm. This region is thought to contain multiple hydrophilic electron donor/acceptor groups, charged groups, and aromatic amino acids, to create a number of subsites where drugs can bind. The flexibility

of the binding pocket would allow induced fit of multiple drugs via hydrophobic interactions, hydrogen bonding, and electrostatic interactions with residues lining the pocket. The number and strength of these interactions would dictate the affinity of drug binding to the protein. In fact, the drug-binding pocket of Pgp contains primarily hydrophobic and aromatic residues, as shown in the crystal structure.[121] The substrate-binding cavity contains 73 solvent-accessible residues, of which 15 are polar and only two are potentially charged. More polar and charged residues are located near the bottom of the drug-binding pocket than in the upper portion, and it is thought that drug substrates carrying a charge will bind such that their charged portions interact with the polar/charged residues in the lower region. The drug-binding pockets of ABCG2 and MRP1 would likely have a similar architecture.

Soluble multidrug-binding proteins have provided insights into how such a polyspecific binding site functions at the molecular level. QacR is a bacterial transcription factor that binds multiple drugs. It has recently been crystallized in complexes with six different drugs, which are located in two overlapping drug-binding regions. These X-ray structures demonstrate the existence of a flexible drug-binding pocket that can accommodate multiple drugs.[182] The binding pocket contains many aromatic amino acids as well as a few polar or charged side chains. Binding occurs primarily via Van der Waal's and hydrophobic interactions, with some polar and electrostatic interactions with hydrophilic or charged groups on the substrates, where possible. QacR can accommodate two different drug molecules at the same time in its binding pocket via induced fit, and the binding mode for each compound may be altered by the presence of other bound drugs.[183] The hydrophobic ligand-binding cavity of the human nuclear pregnane X receptor (PXR), an activator of cytochrome P450 3A expression in response to xenobiotics, was crystallized in the presence of the drug SR12813.[184] The binding cavity contains polar residues that permit SR12813 to bind in three different orientations, where each orientation is stabilized by a different complement of residues. Pgp has a drug-binding pocket large enough to accommodate more than one substrate[185] and was previously shown to bind two different drug molecules simultaneously by fluorescence methods,[186] as well as through binding of a thiol-reactive substrate.[187] In addition, one crystal structure of Pgp shows overlapping binding sites for two stereoisomers of the same drug, and another structure shows two molecules of the same drug located in different regions of the cavity.[121] Thus, Pgp possesses a drug-binding pocket with many features analogous to those of QacR and PXR.

In early attempts to identify the location of the drug-binding sites, Pgp was labeled with photoactive analogues of a variety of drug substrates.[188−193] Identification of the labeled peptides following proteolytic cleavage showed that labeling took place in the TMD of both halves of the protein. More recent work using Cys-reactive substrate analogues and Cys mutations has localized the drug-binding pocket to the regions of Pgp bounded by TM4−6 and 10−12, as residues from these helices are important for binding.[110,111,113,114,194,195] The drug-binding pocket appeared to be funnel-shaped and located at the interface of the two halves of the protein.[115] This interfacial localization was confirmed using propafenone photoaffinity ligands and mass spectrometry, where peptides from TM3, 5, 8, and 11 were specifically labeled.[196] When drugs bind, the packing of helices is altered in Pgp relative to the drug-free state, as shown by Cys cross-linking of pairs of residues.[197] The packing changes are specific to each substrate, which supports an induced-fit model of drug binding.

The most important aspect of the Pgp crystal-structure determination was the successful elucidation of two different structures with novel peptide inhibitors bound to the drug-binding region (Figure 4 parts C−F).[121] The drug-binding site is located within the membrane, with exposure at its sides to the inner leaflet lipid, presumably to allow drug entry from the membrane. It is also open to the cytosol, suggesting that an inward-facing conformation of the protein has been captured. One molecule of the cyclic peptide QZ59-RRR (Figure 4 C and D, green) binds to the "middle" site in the center of the transporter. Interestingly, a second structure of Pgp was solved that contains two bound molecules of QZ59-SSS (Figure 4 E and F, yellow and red), the stereoisomer of QZ59-RRR. One drug molecule occupies an "upper" site within the binding pocket, and the other occupies a "lower" site. Comparison with the QZ59-RRR-bound structure allowed visualization for the first time of Pgp's ability to bind substrate stereoisomers and multiple molecules of the same substrate simultaneously, as previously indicated by substantial biochemical evidence.[186,187,198] QZ59-RRR and QZ59-SSS bind to overlapping regions of the substrate-binding site, and in different orientations, by interacting with a different subset of amino acid residues in the protein (Figure 4 C−F). QZ59-RRR in the middle site binds to primarily hydrophobic residues (such as F, Y, L, and I) on TM1, 5, 6, 7, 11, and 12, although it does also interact with M68, Q721, and S975. The QZ59-SSS molecule in the upper site interacts with hydrophobic residues of TM1, 2, 6, 7, 11, and 12, while in the lower site the drug is in close proximity to residues in TM1 and TM5−12 and is surrounded by Q721, Q986, and S989. The binding of QZ59 to Pgp thus confirms the anticipated importance of TM helices 5−6 and 9−12 in substrate binding, as predicted by cross-linking studies.[199] The Pgp crystal structure suggests that, upon ATP binding/hydrolysis, the drug-binding cavity becomes closed to the inner leaflet and opens to either the outer membrane leaflet or the extracellular solution, in support of the vacuum cleaner and flippase mechanisms of action (see sections 7.1 and 7.2).

MRP1 binds both hydrophobic drugs and free GSH, or drug glutathione conjugates, and each molecule or portion of the conjugate appears to possess a distinct binding site.[200,201] Radiolabeled LTC_4 labels both the N-terminal and C-terminal halves of MRP1, suggesting that there are at least two LTC_4 binding sites.[202] Other work with iodoarylazido-R123 showed that drug substrates bind mainly in TM10−11 and TM16−17, which correspond to TM5−6 and TM11−12 of Pgp.[203] Iodoarylazido-GSH labeling showed that the drug bound not only to hydrophobic TM10−11 and TM16−17 but also to two cytoplasmic linker regions that are polar in nature, suggesting that these regions may be involved in binding of free GSH and glutathione conjugates in a relatively polar environment.[204,205]

The binding site of ABCG2 is expected to share characteristics with those of Pgp and MRP1, but to date far less work has been reported on its characterization. It is expected to be made up of TM helices contributed from each monomer in the homodimer, analogous to the contribution of residues from TM helices in each half of Pgp. Callaghan and coworkers found submicromolar binding affinity for [^{3}H]dauno-

rubicin, which appeared to bind cooperatively, indicating that more than one binding site may be present.[161] While some drugs completely displaced [^{3}H]daunorubicin, others caused only partial displacement, again indicating that ABCG2 contains multiple binding sites that are polyspecific in nature and likely overlap.

6. Catalytic Cycle of ABC Drug Efflux Pumps

6.1. ATP Binding and Hydrolysis

Early work on Pgp using in vitro systems established that drug transport was powered by hydrolysis of ATP, and the same has proved true for MRP1 and ABCG2. All three ABC multidrug efflux pumps display constitutive ATP hydrolysis in the absence of drug substrates; this apparently uncoupled ATPase activity is quite high for Pgp and ABCG2 and substantially lower for MRP1. Pgp and ABCG2 have a relatively high K_m for ATP hydrolysis (0.2−0.5[206,207] and 2 mM,[208] respectively), indicating that their nucleotide binding affinity is low, whereas MRP1 has a much lower K_m of ~100 μM.[209] The affinity and stoichiometry of nucleotide binding to purified Pgp has been quantitated using fluorescent and spin-labeled ATP analogues. The native protein binds two nucleotide molecules[210,211] with a dissociation constant, K_d, of 0.2−0.5 mM,[60] similar to the K_m for ATP hydrolysis. Photoaffinity labeling with azido-ATP analogues was used to examine nucleotide binding to ABCG2[212] and MRP1[213] and revealed that, unlike Pgp, the two NBDs of the latter protein appear to be functionally nonequivalent.

The basal ATPase activity of the transporters is typically modulated by drug substrates; some drugs stimulate activity, others inhibit activity, and many display a biphasic pattern, stimulating activity at low concentrations and inhibiting it at higher concentrations.[214] As yet, there is no satisfactory explanation for this complex behavior. Since drug transport is driven by ATP hydrolysis, there must be conformational communication between the drug-binding pocket and the catalytic site. This was demonstrated by a study in which a fluorescent probe located close to the site of ATP binding displayed a change in its local environment following drug binding to the TM regions of the protein.[60]

The observation that Pgp catalytic activity is rapidly inactivated by addition of the ATPase inhibitor, orthovanadate (V_i), in the presence of ATP led to some remarkable insights into the catalytic cycle of the protein. After a single round of ATP hydrolysis, the P_i analogue is retained in one of the NBDs as the relatively stable complex, ADP·V_i·Mg^{2+}, which is believed to resemble the catalytic transition state structurally.[215] V_i probably occupies the same position as P_i following ATP hydrolysis; thus, the V_i-trapped state represents a posthydrolysis conformation of Pgp. Trapping of vanadate at one active site blocks catalytic turnover at the other site,[216] as does inactivation of one active site by mutation or covalent modification,[217] suggesting that both sites must be functional for ATP turnover to take place. This observation led to the proposal that Pgp operates by an alternating-sites model, whereby only one catalytic site is active at any point in time, and they hydrolyze ATP alternately.[218] Thus, if one site is inactivated, catalysis halts after a single round of ATP turnover. Later work showed that the "vacant" active site in the vanadate-trapped complex of Pgp can bind ATP despite its lack of catalytic turnover.[210] The ATPase activity of MRP1 and ABCG2 is also inhibited by V_i. Given the similarities between Pgp and ABCG2, and the fact that it is a symmetrical dimer, it seems likely that it also operates via a similar alternating-sites mechanism. However, MRP1 may work differently, since its NBDs are structurally and functionally nonequivalent, and V_i trapping appears to take place primarily at the C-terminal NBD.

6.2. Occluded Nucleotide Conformation of Pgp

The stable nucleotide sandwich dimer structures reported for various ABC proteins and isolated NBDs have only been observed when ATP hydrolysis is blocked by either mutation of an essential catalytic residue or the absence of Mg^{2+}, not in situations where the proteins are catalytically active. However, both mutational studies,[217] and the presence of trapped V_i at a single active site,[216] suggest that the two NBDs of Pgp alternate in hydrolysis. This, in turn, implies that the protein must always form asymmetrical structures during catalytic cycling, or "memory" of which of the two active sites last hydrolyzed ATP would be lost. Since the sandwich dimers observed in bacterial ABC protein crystals are symmetrical, they probably do not represent a true catalytic intermediate, at least in the case of drug exporters like Pgp. Tombline and co-workers were the first to isolate an asymmetric nucleotide-bound structure of Pgp by employing the catalytically inactive mutant E552A/E1197A. They found that this protein retained a single molecule of ATP where the binding affinity is approximately 50-fold higher ($K_d \approx 9$ μM) than normally observed ($K_d \approx 2-5$ mM). This nucleotide is observed to be tightly "occluded" within the active site and, unlike loosely bound ATP, cannot be removed by washing or column chromatography. It was later reported that a single molecule of the nonhydrolyzable nucleotide adenosine 5′-(γ-thio)triphosphate (ATPγS) is occluded within wild-type catalytically active Pgp,[219] again suggesting the existence of an asymmetric nucleotide-bound state. More recently, we have used fluorescence spectroscopic approaches to characterize an asymmetric nucleotide-bound state of wild-type Pgp where two molecules of ATPγS are bound, one with the normally observed low affinity (K_d = 0.7 mM) and one with 100-fold higher binding affinity (K_d = 6 μM) (A. Siarheyeva, R. Liu, and F. J. Sharom, unpublished data). ADP and other nonhydrolyzable analogues, including AMP-PNP and adenosine 5′-($\beta\gamma$-methylene)triphosphate (AMP-PCP), are not able to induce the asymmetric state, and both nucleotide molecules are bound with low affinity. The asymmetric intermediate is thought to exist transiently during the normal catalytic cycle, but the tightly bound ATP molecule is committed to hydrolysis and rapidly enters the transition state. It only appears possible to trap the asymmetric intermediate in stable form using ATPγS, or by inactivating an amino acid residue that is required for catalysis.

6.3. Role of NBD Dimerization and the Occluded Conformation in the Catalytic Cycle of Pgp

It is now clear that nucleotide binding to ABC transporters drives dimerization of the NBDs, which is essential for ATP-driven transport. Figure 6 shows a proposed catalytic cycle for Pgp that incorporates what we know about ATP binding stoichiometry and affinity, NBD dimerization, and the occluded state where nucleotide is tightly bound at one of the active sites. The catalytic cycle starts at the upper left, where Pgp contains two ATP molecules, both bound with low affinity (ATP_L). This state is stable and has been

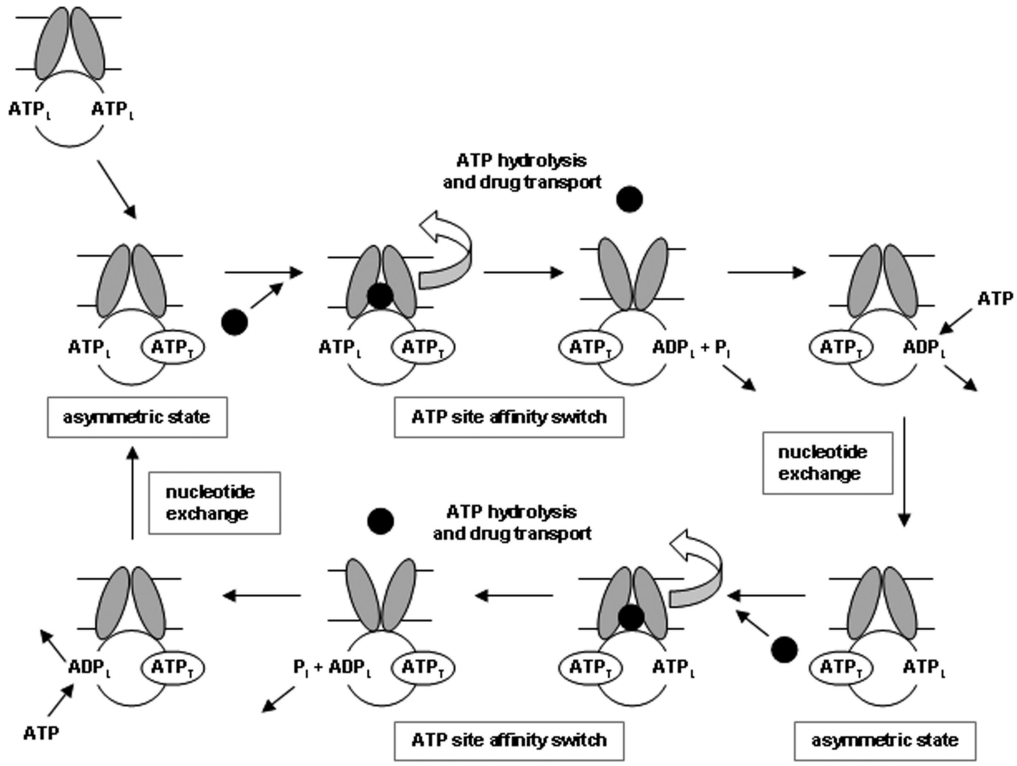

Figure 6. Proposed cycle of NBD dimerization, ATP occlusion, ATP hydrolysis, and drug transport for Pgp. The cycle starts at the upper left, with binding of two molecules of ATP to the pump. If the protein is catalytically inactive, or a nonhydrolyzable ATP analogue such as AMP-PNP is employed, this binding is of relatively low affinity (loosely bound ATP is indicated by ATP_L), with a K_d value of 0.2−0.5 mM.[60,211] The dimer interface is not "closed", as indicated by the break in the half-circle for that NBD. If Pgp is catalytically active, one of the bound ATP molecules rapidly progresses to a tightly bound state (ATP_T), accompanied by "closure" of one-half of the NBD dimer interface, indicated by the oval that now completes the half-circle for that NBD. The tightly bound ATP molecule is committed to hydrolysis and rapidly enters the transition state. Binding of drug (black sphere) to the region of Pgp within the cytoplasmic membrane leaflet is arbitrarily shown here as occurring after ATP binding, but it is known that these two binding steps are not ordered.[60] The tightly bound ATP molecule is then hydrolyzed to ADP and P_i, and the drug is transported to the extracellular environment (or possibly the outer leaflet of the membrane). The protein is assumed to switch from an inward-facing to an outward-facing conformation to effect transport of the drug substrate. The presence of ADP and P_i leads to opening of the closed dimer interface and simultaneous site switching, so that the opposing half of the dimer interface closes around the second ATP molecule, which is now occluded. It is known that P_i leaves the catalytic site first, after which the loosely bound ADP (ADP_L) dissociates and is replaced by another molecule of loosely bound ATP (nucleotide exchange). The asymmetric nucleotide-bound state is thus attained again, but with the tightly bound ATP in the opposing active site committed to hydrolysis. A second identical cycle of catalysis and drug transport is then initiated. During catalytic cycling, at no point does Pgp exist in a symmetric nucleotide-bound state, thus providing "memory" and ensuring that the two active sites alternate in catalysis.

observed in situations where catalysis is blocked: for fluorescently modified Pgp with no catalytic activity bound to native ATP,[60] and for native active Pgp bound to fluorescent[210] or spin-labeled[211] nucleotide analogues that are very poor substrates for hydrolysis by the protein. In this conformation, both halves of the NBD dimer interface are "open", resulting in low ATP binding affinity (K_d of 0.2−0.5 mM). In catalytically active Pgp, the dimer interface rapidly closes around one of the bound ATP molecules, which becomes occluded (ATP_T), resulting in 50- to 100-fold higher binding affinity (K_d of 5−10 μM). This asymmetric nucleotide-bound state of Pgp is normally transient but can be stabilized by the use of the nonhydrolyzable analogue, ATPγS[219] (but not AMP-PNP or AMP-PCP), and by mutation of an essential Glu residue in the catalytic sites (the double mutant E552A/E1197A).[220,221] In native Pgp, the tightly bound ATP molecule is committed to be hydrolyzed and rapidly enters the transition state. The drug to be transported binds to the substrate-binding pocket of Pgp, which is located within the cytoplasmic leaflet of the membrane (see Figure 4 and section 7.2). This step is arbitrarily shown as occurring after ATP loading and formation of the asymmetric occluded state, but it is known that ATP binding and drug binding can take place in any order,[60] and (uncoupled) ATP hydrolysis can take place in the absence of transport substrate. The tightly bound ATP is then hydrolyzed to ADP and P_i, and the transport substrate is moved to either the opposite side of the membrane or the outer leaflet of the bilayer (see sections 7.1 and 7.2). Hydrolysis of ATP at the occluded site results in opening of the dimer interface in that half, likely as a result of electrostatic repulsion between ADP bound to the Walker A motif in one NBD, and P_i bound to the signature C motif of the opposing NBD.[222] Opening of one-half of the NBD dimer interface results in simultaneous "site switching", so that the other half of the dimer interface now becomes closed. The product (ADP_L) is thus loosely bound, and the second ATP molecule now interacts with high affinity and becomes occluded. P_i leaves from the open half of the dimer interface, and nucleotide exchange takes place, so that ADP is replaced by ATP, both loosely bound. At this stage, the protein has attained the asymmetric nucleotide-bound state once again. It reloads with drug, and the steps repeat, with ATP hydrolysis taking place at the other catalytic site. During active cycling, all the reaction intermediates are asymmetric, thus providing "memory". The simultaneous ATP site affinity switch ensures that catalysis alternates between the two NBDs.

Tampé and co-workers have suggested that ABC proteins may work by a processive clamp mechanism,[223] whereby binding of both ATP molecules completely closes both halves of the NBD dimer interface. Both ATPs are hydrolyzed sequentially in the same catalytic cycle (in a processive fashion), which then leads to complete dissociation of both halves of the dimer interface at the end of the cycle. However, such a model predicts the existence of symmetric intermediates, and it is not clear how it can be reconciled with an alternating-sites model. Molecular dynamics simulations predict that both halves of the NBD dimer interface stay in close contact during the catalytic cycle, but only one-half is open at any point in time, in accordance with the alternating-sites proposal.[224,225]

ATP hydrolysis and ATP binding have both been proposed to drive the conformational changes responsible for substrate transport by ABC proteins. Senior and co-workers first proposed that the energy for drug transport by Pgp is provided by relaxation of a high-energy catalytic site conformation generated by ATP hydrolysis.[218] ATP hydrolysis itself, rather than a substrate-binding or product-dissociation reaction, is known to be the rate-limiting step in the catalytic cycle of a bacterial ABC protein,[226] and this is also likely to be true for drug exporters. Trapping experiments showed that P_i leaves the catalytic site first, since it can be replaced by V_i, and this dissociation step was shown to involve a large drop in free energy.[227] This model predicts a concerted mechanism, i.e., that drug transport will take place at the same time as relaxation of the high energy state formed immediately after ATP hydrolysis, for which there is experimental evidence.[228] Higgins and co-workers have proposed the ATP switch model,[229] in which the energy for substrate transport is provided by the free energy of ATP binding. In this model, binding of two molecules of ATP induces the formation of an NBD dimer in which both halves of the interface are in the closed state. ATP hydrolysis is required to reset the transporter to its starting conformation for the next round of catalysis, by dissociating both halves of the NBD dimer into their open form. The ATP switch proposal also predicts the existence of symmetric intermediates, which is incompatible with an alternating-sites model. Clearly, more work will be required to solve the ongoing controversy surrounding the mechanistic details of ATP hydrolysis by ABC transporters.

7. Role of the Membrane in Drug Efflux

7.1. Hydrophobic Vacuum Cleaner Model

Early in the study of MDR, it became evident that classical models for membrane protein solute transport (such as lactose transport by lactose permease) are not a reasonable description for drug efflux processes. Most membrane transporters sequester hydrophilic substrates (sugars, ions, etc.) from the hydrophobic bilayer core and shuttle them across the membrane via a hydrophilic protein pathway that is lined with polar and charged residues. While Pgp, MRP1, and ABCG2 recognize a wide spectrum of compounds (see section 5.1), one feature that is shared, especially for Pgp and ABCG2, is the relative hydrophobicity of their transport substrates. Thus, many drugs that are substrates for these drug efflux pumps can readily cross lipid bilayers by passive diffusion.

Higgins and Gottesman proposed a "hydrophobic vacuum cleaner" model[230] to account for the lipophilic nature of Pgp substrates (Figure 7A). They suggested that drugs bind to Pgp after they have partitioned into the bilayer, and the protein "sucks" them out of the membrane and expels them into the extracellular aqueous phase. There is substantial experimental evidence to support this model, and it is widely accepted. When the lipophilic probe iodonaphthalene-1-azide was used to photolabel Pgp, fluorescence resonance energy transfer data showed that the substrate doxorubicin was present within the membrane in close proximity to the transporter, rather than inside the cell.[231] This suggested that Pgp may bind doxorubicin from within the membrane and extrude it from there to the cell exterior. When acetoxymethyl esters of fluorescent dyes are added to intact cells, Pgp intercepts them before they can come into contact with cytosolic esterases and expels them into the extracellular medium.[232] The dye H33342 only becomes fluorescent after

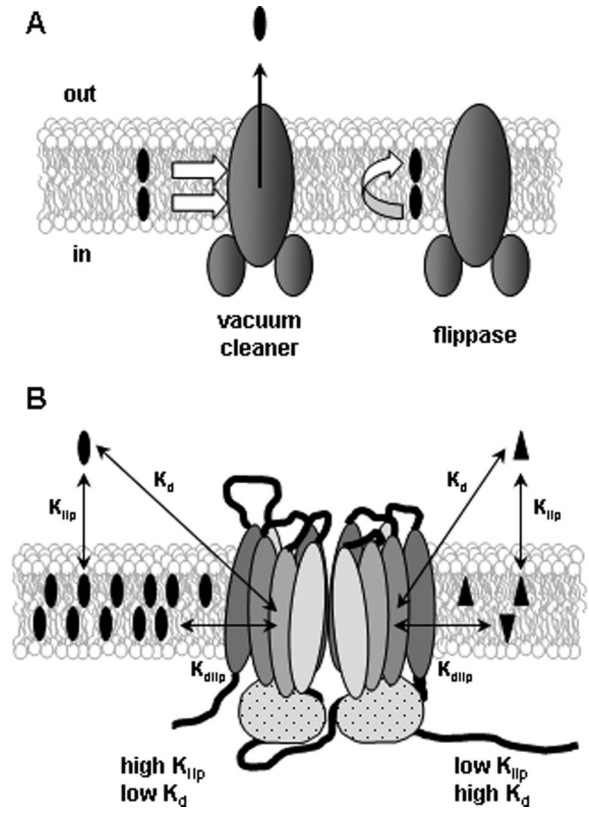

Figure 7. (A) Hydrophobic vacuum cleaner and flippase models for the action of multidrug efflux pumps. The transporter is proposed to interact with its substrates within the membrane and either expels them to the aqueous external environment (vacuum cleaner) or translocates them from the cytoplasmic leaflet to the extracellular leaflet (flippase). (B) Substrate binding to multidrug efflux pumps from within the membrane. Drugs partition from the aqueous phase into the lipid bilayer in accordance with their lipid−water partition coefficients (K_{lip}). The drug on the left of the diagram has a high K_{lip} value and, thus, reaches a high concentration in the lipid phase, whereas the drug on the right of the diagram has a low K_{lip} value and has a much lower membrane concentration. Typical K_{lip} values for Pgp substrates are in the range 300−20 000. The apparent binding affinity (K_d) is determined using aqueous drug concentrations and is the product of both drug partitioning into the lipid phase and drug binding to the transporter from the membrane. The intrinsic binding affinity (K_{dlip}) of the efflux pump is estimated using the drug concentrations in the lipid phase and is relatively low. A drug with a high K_{lip} value will appear to have a low K_d value (i.e., higher binding affinity) compared to a drug with a low K_{lip} value, even though they have the same intrinsic binding affinity (K_{dlip}). This is because the drug with a higher K_{lip} value will reach a much greater concentration in the membrane.

partitioning into the hydrophobic membrane interior, and kinetic measurements showed that its rate of transport by Pgp was directly proportional to its concentration in the lipid phase, rather than the aqueous medium.[168] Crystallographic data now show that the Pgp drug-binding pocket is indeed accessible via the inner leaflet of the membrane.[121] Because many Pgp substrates are also recognized by ABCG2, this half-transporter likely also binds drugs from within the membrane. MRP1 binds more soluble drug conjugates and might not operate as a hydrophobic vacuum cleaner. However, this efflux pump generally transports both a hydrophobic substrate and GSH (or a glutathione-substrate conjugate),[200] and a hydrophobic binding site for drug and a hydrophilic binding site for GSH may both exist within the protein (see section 5.1).[233] Since photoreactive substrates such as iodoarylazido-R123 label the relatively hydrophobic TM10−11 and TM16−17 regions of MRP1,[203] the process of binding the hydrophobic substrate may in fact involve the membrane, as it does for Pgp and ABCG2.

7.2. Flippase Model

The original proposal of the hydrophobic vacuum cleaner model suggested that Pgp may act as a translocase or "flippase" that moves its substrates from the cytoplasmic leaflet to the extracellular leaflet of the bilayer, rather than into the aqueous phase (Figure 7A).[230] This would result in a higher drug concentration in the outer leaflet compared to the inner leaflet. Since substrate will partition between the outer membrane leaflet and the extracellular medium, and between the inner membrane leaflet and the cytoplasm, a drug concentration gradient across the membrane would be observed. The vacuum cleaner and flippase models are not mutually exclusive. At the time of substrate release, the binding site could become exposed to both the extracellular leaflet and the extracellular medium, and it is possible that substrates are released into either of these locations, depending on their hydrophobicity. The greatest energy barrier to efflux would be the rehydration of a hydrophobic substrate, suggesting that, in general, release of drug into the outer leaflet lipids would be most favorable. However, it is very difficult to distinguish experimentally between translocation of drug directly to the extracellular leaflet and transport to the extracellular medium followed by rapid repartitioning into the membrane, since the same equilibrium state is reached.

The flippase model requires that drug substrates localize to one leaflet of the bilayer, rather than to the hydrophobic core of the membrane. It also necessitates a low rate of spontaneous movement of substrates between the two bilayer leaflets to allow Pgp to generate a drug concentration gradient. This appears to be the case. A panel of 9 molecules that bind to Pgp were shown to distribute discretely in one membrane leaflet, where they were localized to the interfacial region in a similar orientation to phospholipids.[234] Additionally, the rate of movement of many Pgp substrates across a lipid bilayer ranges from minutes to hours,[235] and lipids, some of which appear to be Pgp substrates (see below), have a flip-flop half-time of hours to days.

In support of the flippase model, several ABC proteins appear to be involved in translocation of phospholipids and their derivatives. The *Lactococcus lactis* ABC half transporter, LmrA, which is homologous to mammalian ABCG2 and Pgp[236] and has ATP-dependent drug efflux activity,[237] displays ATP-dependent flippase activity with fluorescently labeled PE, but not phosphatidylcholine (PC).[238] The bacterial protein MsbA is proposed to be a lipid flippase for the lipopolysaccharide precursor lipid A, yet it also binds[239,240] and transports[241] Pgp substrates and flips fluorescently labeled phospholipids and glycolipids (P. D. W. Eckford and F. J. Sharom, unpublished data). The yeast ABC transporters Yor1P and Pdr5p, and the *Candida* drug resistance protein 1 (Cdr1p), have all been shown to transport/flip fluorescent phospholipids.[242,243]

Both phospholipids and lipid-derived signaling molecules, such as C_{16} platelet-activating factor (PAF-16), interact directly with Pgp and may be endogenous substrates.[167] Hexadecylphosphocholine (miltefosine), a drug structurally related to PC, appears to be transported by Pgp,[167,244] and other lipid-based anticancer drugs also interact with the protein.[167] While Pgp is a known drug transporter, the product of the closely related ABCB4 gene is not a multidrug-resistance protein, but rather a PC-specific flippase that exports this phospholipid from the hepatocyte canalicular membrane into the bile.[245−247] The two proteins have high sequence similarity (78%), and ABCB4 can transport drug substrates at a low rate.[248]

Pgp appears to function as a phospholipid flippase as well as a drug transporter. Altered distributions of fluorescent PC, PE, and sphingomyelin (SM) derivatives were found in cells expressing recombinant Pgp[249] or drug-selected cells overexpressing the protein.[250] Both short-chain[249] and long-chain[251] fluorescent phospholipids were found to accumulate to a lesser extent in Pgp-expressing cells, and accumulation was increased upon treatment with a Pgp modulator.[250] Using purified Pgp reconstituted into proteoliposomes, we have shown directly that the protein can flip a variety of fluorescently labeled phospholipids and glycosphingolipids in an ATP-dependent, vanadate-sensitive fashion.[51,54] Phospholipid and glycosphingolipid translocation was inhibited in a concentration-dependent manner by known Pgp substrates, and inhibitory potency was highly correlated with their Pgp binding affinity.[51,54]

Both biochemical and structural data indicate that the substrate binding pocket of Pgp is located within the TM regions of the protein that contact the cytoplasmic membrane leaflet,[118,119,121,252] which is consistent with it acting as either a vacuum cleaner or a flippase. It was recently suggested that substrates enter the drug-binding pocket from the membrane through "gates" formed by the cytoplasmic ends of TM5/8 and TM2/11 in each half of the transporter. Labeling studies have indicated that the Pgp drug-binding pocket is accessible to the aqueous medium,[253] although this is contradicted by work using fluorescent substrates, which indicated that the binding pocket is relatively hydrophobic, with a polarity lower than that of chloroform.[119] The bound drug molecules in the Pgp crystal structure are contained within the membrane, although the apparent openness of the structure could make them water-accessible. However, the in vivo structure of the transporter might be more compact, with the two NBDs closely associated. ATP hydrolysis presumably closes off the Pgp drug-binding pocket from access to the inner membrane leaflet and opens up access to either the extracellular leaflet or the external aqueous medium, where the substrate would have to be rehydrated at an energy cost.

ABCG2 is a member of a family of cholesterol-transport proteins, and while it appears to primarily function as a drug efflux pump, the protein can also transport sulfate conju-

gates,[146,147] glucuronide conjugates,[254] and unconjugated[255−257] bile acids and steroids. ABCG2-expressing cell lines show enhanced PS exposure and outward transport of fluorescently labeled PS and PC, which is sensitive to both protein expression level and the inhibitor tryprostatin A.[258] ABCG2 may also play a role in membrane lipid leaflet distributions in trophoblast cells.[259] It seems likely that ABCG2 interacts with its substrates in a similar manner to Pgp, according to the vacuum cleaner or flippase models.

MRP1 has also been shown to transport a variety of fluorescent lipids, including GlcCer, SM, PS, and PC.[260−262] Dekkers et al. showed that fluorescently labeled and natural PC and SM accumulated to a greater degree in the inner leaflet of the erythrocyte membrane in the presence of the MRP1 inhibitors verapamil and indomethacin, compared to control cells.[263] MRP1 has also been implicated in protection from the toxic lipid peroxidation product 4-hydroxynonenal.[264] More recently, MRP1 was shown to function as a phospholipid flippase for fluorescent PC in a reconstituted system.[265] However, because of its preference for more soluble drug conjugates, it is not known whether MRP1 plays a significant physiological role in transport of lipids other than C_4 leukotriene,[266] or if a vacuum cleaner or flippase mechanism is used. Perhaps cotransported GSH is transported from a hydrophilic binding site to the external aqueous medium, while more hydrophobic substrates, or the hydrophobic portion of a drug conjugate, might occupy a less polar site within the membrane.

7.3. Membrane Partitioning of Drugs

Many Pgp and ABCG2 substrates display high lipid bilayer−water partition coefficients (K_{lip}),[267−269] and such partitioning is known to be dependent upon the lipid composition of the membrane.[268,270] Measurement of K_{lip} values thus indicates that the membrane effectively concentrates substrates by 300- to 20 000-fold relative to their concentration in aqueous solution.[268,271] The dissociation constant, K_d, for binding of aqueous substrate to the drug pump (which can be thought of as an apparent binding affinity) thus represents the product of two processes: partitioning of substrate into the lipid bilayer (as described by K_{lip}) and subsequent binding of substrate to the transporter from within the bilayer (as described by the parameter K_{dlip}; see Figure 7B). For Pgp and ABCG2, which likely bind their substrates from within the membrane, the drug concentration that the transporter actually "sees" is orders of magnitude higher than the aqueous concentration (Figure 7B). The intrinsic drug-binding affinity of these pumps can be calculated as $K_{dlip} = K_d \times K_{lip}$ (see Figure 7B) and may, therefore, be quite low. For reconstituted Pgp, we reported that K_d, as determined by fluorescence quenching, is correlated with the value of K_{lip} for several drugs in three different lipid systems.[268] The highest apparent binding affinity was observed for substrates that had the greatest partitioning into lipid. More recently, we have confirmed these results using a panel of structurally related rhodamine dyes (A. G. Dyer, P. D. W. Eckford, and F. J. Sharom, unpublished data). We observed apparent Pgp binding affinities (K_d values) of 0.2−15 μM and calculated relatively low intrinsic affinities (K_{dlip} values) of 0.22−12 mM for drug binding to the protein from within the membrane. A thermodynamic analysis of drug binding to Pgp within the lipid bilayer confirms these ideas.[272] The relationship between the membrane partitioning and binding affinity of Pgp substrates suggests one way to reduce or circumvent drug resistance. If a chemotherapeutic drug can be chemically modified to reduce its lipophilicity, this might reduce the ability of Pgp to transport it. This would allow the drug to reach its intracellular targets, and thus, it would exhibit increased clinical effectiveness.

8. Modulation of Drug Efflux Pumps in Chemotherapy Treatment

8.1. Modulators of Drug Efflux Pumps

A variety of compounds have been identified, known as modulators, reversers, inhibitors, or chemosensitizers, that can reverse MDR mediated by the ABC multidrug efflux pumps. Modulators are able to reverse MDR in intact cells in vitro by interfering with the ability of the transporter to efflux drugs. Modulators generally do not kill MDR cells directly, but when they are coadministered with a cytotoxic drug, they restore cytotoxicity. Efflux of the drug is blocked, and it is observed that its LD_{50} shifts to a lower value in the presence of modulator, so the cell is killed. Some of them (the so-called classical modulators) appear to interact with the substrate-binding pocket of the protein and compete with cytotoxic drugs for transport. Indeed, many of these compounds are themselves transported. Modulators are of clinical interest because they have the potential to prevent MDR in chemotherapeutic drug treatment, improve drug uptake in the intestine, and allow drug delivery to protected tissues such as the brain. Discovery of the first modulators was serendipitous, but in recent years combinatorial chemistry, drug design, and the use of protein structural information have all played increasingly important roles in identifying more effective compounds.[273]

Modulators of ABC multidrug efflux pumps are as structurally diverse as substrates,[274] sharing many of their characteristics (Figure 8). Pgp modulators include calcium channel blockers, calmodulin antagonists, cyclic peptides, steroids, and others (Table 2). ABCG2 is inhibited specifically by modulators such as pantoprazole, fumitremorgin C, and derivatives such as Ko132, Ko134, and Ko143 and shares modulators such as elacridar and tariquidar with Pgp. Modulators of MRP1 have been more difficult to identify. The LTC_4 analogue MK571, S-decylglutathione, and probenecid have been described as modulators.[80,275,276] All three ABC drug efflux pumps interact with the modulator VX-710 (biricodar; Table 2).

The way in which modulators act at the molecular level is still not well-understood. The action of classical modulators can be explained using the flippase model. Both substrates and modulators bind to Pgp,[60,157] and at least some modulators, such as cyclosporin A, verapamil, and trans-flupentixol, are known to be transported (reviewed in ref 133). It has been proposed that Pgp modulators may be distinguished from substrates by a high intrinsic rate of trans-bilayer movement.[235] Substrates (with low rates of spontaneous flip-flop) would bind to the protein within the cytoplasmic leaflet of the bilayer and be transported to the extracellular leaflet faster than their intrinsic flip-flop rate. Thus, Pgp would maintain different drug concentrations in the two bilayer leaflets (higher in the outer leaflet), which would lead to the generation of a drug concentration gradient across the membrane. Addition of a modulator would perturb the system as follows: after partitioning into the outer leaflet, the modulator would rapidly translocate spontaneously to the

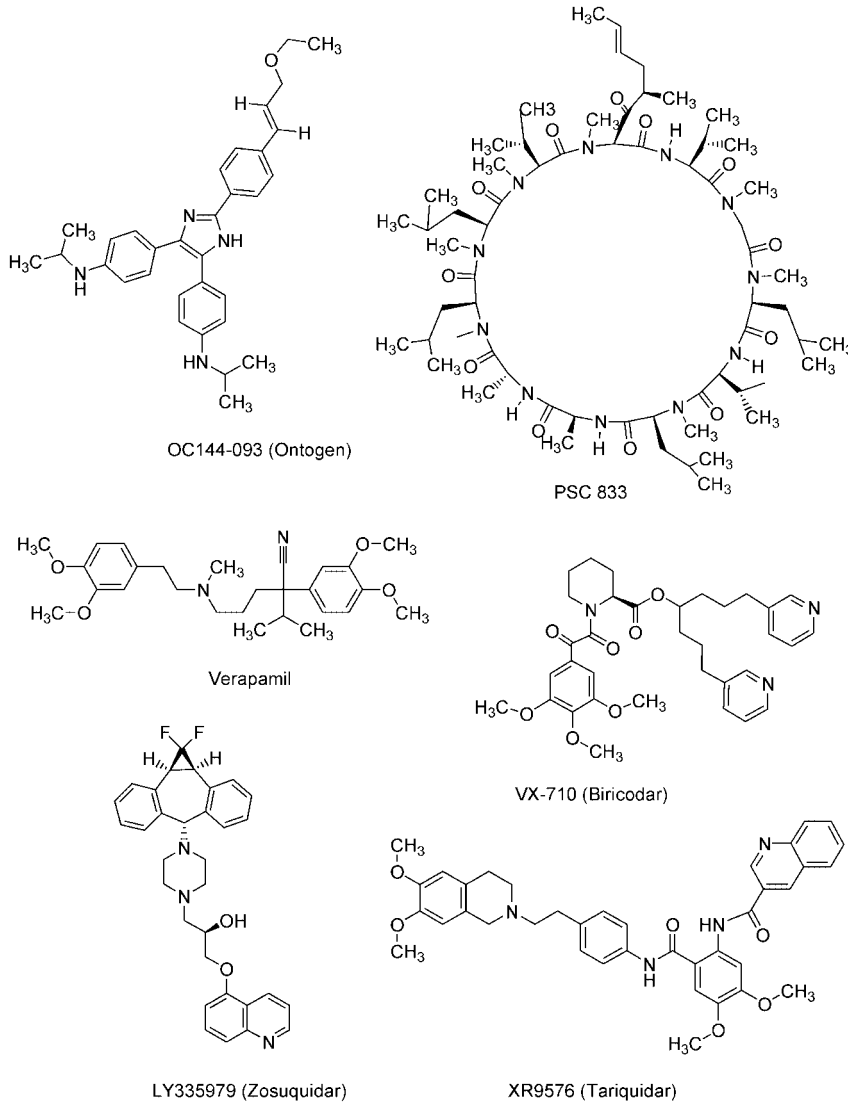

Figure 8. Structures of some modulators that inhibit drug transport and reverse MDR mediated by Pgp, MRP1, and ABCG2.

inner leaflet, where it would be recognized as a substrate and transported back to the outer leaflet. Once in the outer leaflet, the modulator would once again rapidly flip to the inner leaflet, thus locking Pgp in a futile cycle of modulator transport and ATP hydrolysis. This increased rate of ATP turnover has been observed for Pgp-expressing MDR cells treated with the modulator verapamil.[277] A concentration gradient would not be established for the modulator because of its rapid rate of transbilayer movement. Because Pgp would be locked into a futile cycle with the modulator, the cytotoxic drug would be transported at a much lower rate, thus gaining access to the cell interior and causing cell death more effectively. In support of this model, Eytan et al. reported rapid transbilayer movement for several Pgp modulators (e.g., quinidine and quinine), which equilibrated at rates too fast to be determined, but much slower flip-flop rates for substrates (e.g., R123), which equilibrated with a half-time of 3 min.[235] The effect of some modulators on ABCG2 and MRP1 may also be mediated by this mechanism.

However, not all Pgp modulators appear to be transported. For example, LY335979 (zosuquidar) displays prolonged, very high affinity binding to the protein, suggesting that it binds very tightly to the drug-binding pocket and, in this way, blocks transport of drug substrates.[278] Other modulators may inhibit pump-based resistance by a mechanism different from that of classical modulators. For example, the high affinity modulator XR9576 appears to bind at sites distinct from the drug-binding pocket and likely exerts a direct inhibitory effect on ATP-hydrolysis.[153] Several hydrophobic steroid modulators appear to bind to a site within the NBDs and are not transported by Pgp.[279] Disulfiram is known to bind to Pgp and MRP1 at the NBDs as well as the TMDs and likely exerts its inhibitory effect in part by covalently modifying a catalytic residue.[280] Membrane fluidizers (e.g., Nonidet P40), anesthetics (e.g., benzyl alcohol), and the surfactant vehicles Cremophor EL and Solutol HS15[281] can also reverse Pgp-mediated MDR. They are not believed to interact directly with the protein, but rather they may act nonspecifically to increase the intrinsic flip-flop rate of drug substrates in the membrane, thus allowing them to overwhelm the drug pump and effectively restore cytotoxicity.[235,282,283] Verapamil and one of its derivatives have been shown to modulate MDR in cells expressing functional MRP1 by another mechanism, induction of apoptosis by extrusion of GSH.[284] This apoptotic process is dependent on functional MRP1[284,285] and can be modulated by control of cellular GSH levels.[286] Induction of apoptosis by treatment with modulators has also been demonstrated for Pgp-expressing cells and occurs by an unknown mechanism possibly related to failure of cytokinesis for modulators such

Table 2. Modulators That Interact with ABC Multidrug Efflux Pumps

Pgp	MRP1	ABCG2
first-generation modulators	VX-710 (biricodar)	***flavonoids***
verapamil	MK571	silymarin
cyclosporin A	probenecid	hesperetin
	S-decylglutathione	diadzein
		biochanin A
		chrysin
		tectochrysin
second-generation modulators		6-prenylchrysin
PSC-833 (valspodar)		apigenin
VX-710 (biricodar)		3′,4′,7-trimethoxyflavone
		quercetin
third-generation modulators		***fumitremorgin C analogues***
LY335979 (zosuquidar)		fumitremorgin C
XR9576 (tariquidar)		Ko132
GF120918 (elacridar)		Ko134
OC144−093 (ontogen)		Ko143
		tryprostatin A
others		***tyrosine kinase inhibitors***
benzyl alcohol		gefitinib
disulfiram		imatinib
Nonidet P40		EKI-785
Cremaphor EL		CI1033
curcuminoids		tRA98006
plant flavonoids		***others***
		GF120918 (elacridar)
		VX-710 (biricodar)
		XR9576 (tariquidar)
		nelfinavir
		lopinavir
		novobiocin
		pantoprazole

as LY335979 and PSC-833,[287] or through production of reactive oxygen species as a result of Pgp-mediated ATP turnover stimulated by verapamil or other drugs.[288]

8.2. Modulator Treatment of MDR Cancers

The paradoxical nature of treatment with modulators for overcoming efflux pump-mediated MDR is 2-fold. The protein's drug transport activity must be inhibited to sensitize MDR cancers, which involves administration of a modulator in conjunction with a cytotoxic chemotherapeutic drug. However, inhibition of the drug pump leaves sensitive tissues, including the brain, susceptible to harm by cytotoxic compounds administered to kill the tumor cells. Altered drug clearance is also a major concern, since many modulators are also known to be substrates for the cytochrome P450 enzyme (CYP450 3A) that is involved in metabolism and elimination of therapeutic drugs. Reduced drug clearance leads to increased toxicity, a serious problem. Additionally, administration of efflux pump substrates and modulators may upregulate expression of these proteins,[289] which risks making the tumor cells more drug-resistant and, thus, more difficult to treat. Expression of multiple drug efflux pumps and the existence of other resistance mechanisms in clinical MDR have increased uncertainty as to whether modulators will increase patient survival, and this subject is still controversial.[26] Thus, treatment of cancer patients with modulators is a complex process, and the results of clinical trials have thus far been disappointing.[290]

First-generation modulators of Pgp included compounds like verapamil (a calcium channel blocker) and cyclosporin A (an immunosuppressant)(Figure 8), which were already employed to treat other medical conditions, but also blocked drug pump function in MDR cell lines in vitro.[291,292] However, when used in clinical trials, these molecules generally caused excessively high toxicity yet showed low effectiveness at doses that were tolerated by patients.[2] To address these issues, second-generation modulators were developed; these were often derivatives of first-generation molecules, such as PSC-833, a cyclosporin A derivative. Second-generation molecules displayed higher affinities for binding to the drug pump and, thus, better efficacy at low doses. However, significant increases in toxicity and decreased clearance of the chemotherapeutic drug were often observed as a result of CYP450 3A inhibition, necessitating a dose reduction.[293−295] Third-generation modulators were developed to improve on the properties of the second-generation compounds. Some highly selective and very potent inhibitors were produced, which are effective in the nanomolar concentration range; they include GF120918[296] (elacridar), LY335979[297] (zosuquidar), XR9576[298] (tariquidar), and OC144-093[299] (ontogen) (Table 2 and Figure 8). These molecules show promise in cancer treatment and are currently in clinical trials (reviewed in ref 300). To date, little work has been done to examine the effects of specific modulation of MRP1 and ABCG2 in clinical trials. However, some Pgp modulators such as VX-710 show cross-reactivity with the other two efflux pumps, suggesting that tumor cells expressing a combination of drug efflux pumps might respond to treatment with these compounds.

Several natural products of relatively low toxicity, including curcumin,[301] are reported to be modulators of one or more ABC drug efflux pumps, and there is a wealth of literature demonstrating the modulatory effects of plant flavonoids. The flavonoids are a group of molecules of broad structural diversity that were originally isolated from citrus fruits and include isoflavones, flavones, flavanones and their chemically synthesized analogues. A variety of flavonoid molecules have been shown to modulate Pgp activity in vitro[302−304] and in cultured cells.[305−311] Some flavonoids have also been shown to have modulatory effects on both MRP1[312−316] and ABCG2,

for which specific new modulators have been identified[317−321] (see Table 2). As yet, the potential clinical use of such natural products has not been satisfactorily explored.

9. Future Directions

Over the past 5 years, structural information on bacterial ABC transporters (primarily importers) has been emerging rapidly, leading to advances in our knowledge of how these proteins work at the molecular level, both in terms of the ATP hydrolysis cycle and coupled drug transport. With the publication of the first high-resolution structures of Pgp, we now have a rudimentary understanding of the nature of the multidrug-binding pocket of this transporter. Further high-resolution information is needed on all three mammalian efflux pumps, including nucleotide-bound structures, and structures with drugs bound alone and in combination. This will allow us to move forward in designing specific inhibitors for clinical use in reversing MDR in cancer patients.

10. Acknowledgments

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11. Abbreviations

ABC	ATP-binding cassette
ABCP	placenta-specific ABC transporter
ALL	acute lymphoblastic leukemia
AML	acute myelogenous leukemia
AMP-PCP	adenosine 5′-($\beta\gamma$-methylene)triphosphate
AMP-PNP	adenosine 5′-($\beta\gamma$-imido)triphosphate
ATPγS	adenosine 5′-(γ-thio)triphosphate
CLL	chronic lymphocytic leukemia
BCRP	breast cancer resistance protein
GlcCer	glucosylceramide
GSH	reduced glutathione
GSSG	oxidized glutathione
H33342	Hoechst 33342
HIV	human immunodeficiency virus
LTC_4	leukotriene C_4
MDR	multidrug resistance/resistant
^{99m}Tc-MIBI	technetium-99m methoxyisobutylisonitrile
MXR	mitoxantrone resistance protein
MRP	multidrug resistance-associated protein
NBD	nucleotide-binding domain
PAF	platelet-activating factor
PC	phosphatidylcholine
PE	phosphatidylethanolamine
Pgp	P-glycoprotein
PS	phosphatidylserine
PXR	pregnane X receptor
R123	rhodamine 123
SM	sphingomyelin
TM	transmembrane
TMD	transmembrane domain
TMR	tetramethylrosamine

12. References

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