REVIEW

ABC multidrug transporters: structure, function and role in chemoresistance

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Three ATP-binding cassette (ABC)-superfamily multidrug efflux pumps are known to be responsible for chemoresistance; P-glycoprotein (ABCB1), MRP1 (ABCC1) and ABCG2 (BCRP). These transporters play an important role in normal physiology by protecting tissues from toxic xenobiotics and endogenous metabolites. Hydrophobic amphipathic compounds, including many clinically used drugs, interact with the substrate-binding pocket of these proteins via flexible hydrophobic and H-bonding interactions. These efflux pumps are expressed in many human tumors, where they likely contribute to resistance to chemotherapy treatment. However, the use of efflux-pump modulators in clinical cancer treatment has proved disappointing. Single nucleotide polymorphisms in ABC drug-efflux pumps may play a role in responses to drug therapy and disease susceptibility. The effect of various genotypes and haplotypes on the expression and function of these proteins is not yet clear, and their true impact remains controversial.

Keywords: ABCG2 (BCRP), ABC superfamily, ATP hydrolysis, drug efflux, drug therapy, MRP1/ABCC1, multidrug resistance, P-glycoprotein/MDR1/ ABCB1, polymorphisms, transport mechanism

The ATP-binding cassette (ABC) superfamily of proteins is one of the largest protein families in biology [1]. It consists largely of membrane proteins that transport a diverse array of substrates, including sugars, amino acids, drugs, antibiotics, toxins, lipids, sterols, bile salts, peptides, nucleotides, endogenous metabolites and ions. ABC proteins are present in the cytoplasmic (inner) membrane of bacteria, and in both the plasma membrane and organelle membranes in eukaryotes. The human genome encodes 49 ABC proteins [2,3], only a fraction of which have been characterized in terms of their biochemistry and function. They have been classified into seven subfamilies based on phylogenetic analysis [2]. ABC proteins in their functional form comprize a minimum of four core domains; two membrane-bound domains that form the permeation pathway for transport of substrates, and two nucleotide binding domains (NBDs) that hydrolyze ATP to power this process. In bacteria, these four domains exist as two or four separate polypeptides, whereas in eukaryotes, the four domains are often fused into a single large protein with an internal duplication. Proteins in the ABCC subfamily possess an extra N-terminal transmembrane (TM) domain of unknown function. While prokaryotic ABC proteins can be either importers or exporters, eukaryotic family members are exclusively exporters. ABC proteins are active transporters, pumping their substrates up a concentration gradient using the energy of ATP hydrolysis.

Mammalian ABC proteins have gained prominence as their involvement in maintaining human health became evident; a total of 14 human ABC transporters have now been associated with a specific disease state [4]. In some cases, the physiological substrate is known (e.g., ABCB11 transports bile salts; and loss-of-function mutations produce progressive familial intrahepatic cholestasis type 2 [PFIC-2]), whereas for some proteins it remains to be determined (e.g., the disease *Pseudoxanthoma elasticum* is caused by loss-of-function mutations in *ABCC6*, whose physiological substrate has not yet been identified). Several ABC proteins are multidrug efflux pumps that not only protect the body from exogenous toxins, but also play an important role in the uptake and distribution of therapeutic drugs [5]. They can, therefore, profoundly affect drug therapy, and resistance to treatment by multiple drugs has been associated with their expression in the target tissue. For example, multidrug resistance (MDR) to chemotherapeutic drugs is a serious barrier to successful treatment of many human cancers. Polymorphisms in ABC drug transporters have been increasingly studied over the past few years, since it seems likely that they will be responsible for varying responses to drug therapy in the population. This review focuses on what we currently know of the molecular structure, substrates, transport mechanism and polymorphisms of the three most important ABC multidrug transporters, and discusses their role in drug resistance in clinical therapy.

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ABC multidrug transporters

The role of ABC proteins in resistance to anticancer drugs has been known for over 30 years [6]. A total of 15 family members can function as drug-efflux pumps, and have been implicated in potentially conferring resistance to chemotherapeutic agents (see [5,7] for reviews). However, three ABC proteins appear to account for most observed MDR in humans and rodents; P-glycoprotein (Pgp/MDR1/ABCB1), MDR-associated protein (MRP)1 (ABCC1) and breast cancer resistance protein ABCG2 (variously known as BCRP, ABCP or MXR) [8]. The year 2006 represented the 30th anniversary of the discovery of Pgp. These drug transporters are located in the plasma membrane, where they engage in active efflux of drugs and drug conjugates. Pgp and MRP1 are 170–190 kDa single polypeptides, while ABCG2 is a 72 kDa half-transporter, and likely functions as a homodimeric complex [9]. Pgp is the mammalian ABC protein that we know most about in terms of its structure and mechanism. It has been described as a 'double-edged sword', in that it protects sensitive tissues from potentially toxic xenobiotics and yet also causes MDR in tumors, thus preventing effective chemotherapeutic treatment. ABCG2 and MRP1 also share many of these features with Pgp.

Pgp and ABCG2 can export both unmodified drugs and drug conjugates, whereas MRP1 exports glutathione and other drug conjugates, and unconjugated drugs together with free glutathione. All three demonstrate overlapping drug specificity. This redundancy indicates that a complex network of efflux pumps is involved in protecting the body from toxic xenobiotics. Pgp transports a wide range of structurally dissimilar compounds, many of which are clinically important, including anticancer drugs, HIV-protease inhibitors, analgesics, antihistamines, H_2-receptor antagonists, immunosuppressive agents, cardiac glycosides, calcium-channel blockers, calmodulin antagonists, antiemetics, antihelminthics, antibiotics, steroids (see Box 1 for a list of selected compounds that interact with Pgp). All are amphipathic, lipid-soluble compounds, with molecular weights in the range of 300 to 1000, often with aromatic rings and a positive charge at physiological pH. Physiological substrates for Pgp potentially include steroid hormones, lipids, peptides and small cytokines. To date, endogenous biomolecules that have been identified as likely Pgp substrates include several phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin) [10–12], simple glycosphingolipids (glucosylceramide, galactosylceramide and lactosylceramide) [12], platelet-activating factors [13,14], aldosterone [15], β-estradiol-17β-D-glucuronide [16], β-amyloid peptides [17] and several interleukins [18]. The *ABCB4* gene product is a very close relative, with 78% sequence homology to ABCB1. This Pgp isoform functions to export phosphatidylcholine from the liver canalicular cells into the bile, and is believed to be a lipid flippase [19], although it can also transport drugs with low efficiency [20].

MRP1 transports a variety of endogenous molecules of physiological significance [21], including free glutathione, glutathione-conjugated leukotrienes and prostaglandins (LTC_4, LTD_4 and LTE_4, prostaglandin A_2-SG, hydroxynonenal-SG), glucuronide conjugates (β-estradiol-β-D-glucuronide and glucuronosyl-bilirubin) and sulfate conjugates (dehydroepiandrosterone-3-sulfate and sulfatolithocholyl-taurine) (Box 2). Similarly to Pgp, MRP1 also confers resistance to a variety of anti-cancer agents, although not taxols. MRP1 prefers anionic substrates, and drugs are either exported as anionic glutathione, glucuronate or sulfate conjugates, or cotransported with free glutathione. MRP1 also transports heavy metal oxyanions such as arsenite and trivalent antimonite [22].

ABCG2 is a broad specificity drug transporter like Pgp, however it appears to transport both positively and negatively charged drugs, including sulfate conjugates (Box 3) [23,24]. It was first discovered based on its ability to transport mitoxantrone, which is a poor substrate for Pgp and MRP1. ABCG2 cannot transport taxols, cisplatin and verapamil (Pgp substrates), calcein (an MRP1 substrate) or *Vinca* alkaloids and anthracyclines (substrates for both Pgp and MRP1), indicating that its substrate specificity partially overlaps with that of the other two transporters. ABCG2 transports both Gleevec® (imatinib) and Iressa® (gefitinib), two recently introduced anticancer drugs that are tyrosine kinase inhibitors; these compounds also interact with Pgp and ABCC1, but substantially higher concentrations are required. The list of ABCG2 substrates is rapidly expanding, highlighting the importance of this protein.

All three ABC drug-efflux pumps are able to transport fluorescent compounds (Boxes 1, 2 and 3). These have proved very useful as a tool for exploring the transport activity of the ABC proteins in intact cells. For example, quantitation of

Box 1. Clinically relevant drugs and other compounds that interact with P-glycoprotein (ABCB1).

Anticancer drugs
- *Vinca* alkaloids (vinblastine and vincristine)
- Anthracyclines (doxorubicin and daunorubicin)
- Taxanes (paclitaxel and docetaxel)
- Epipodophyllotoxins (etoposide and teniposide)
- Camptothecins (topotecan)
- Anthracenes (bisantrene and mitoxantrone)

HIV protease inhibitors
- Ritonavir
- Saquinavir
- Nelfinavir

Analgesics
- Morphine

Antihistamines
- Terfenadine
- Fexofenadine

H_2-receptor antagonists
- Cimetidine

Immunosuppressive agents
- Cyclosporine A
- Tacrolimus (FK506)

Antiarrhythmics
- Quinidine
- Amiodarone
- Propafenone

Antiepileptics
- Felbamate
- Topiramate

Fluorescent compounds
- Calcein-AM
- Hoechst 33342
- Rhodamine 123

HMG-CoA reductase inhibitors
- Lovastatin
- Simvastatin

Antiemetics
- Ondansetron

Tyrosine kinase inhibitors
- Imatinib mesylate
- Gefitinib

Cardiac glycosides
- Digoxin

Antihelminthics
- Ivermectin

Calcium-channel blockers
- Verapamil
- Nifedipine
- Azidopine
- Diltiazem

Calmodulin antagonists
- Trifluoperazine
- Chlorpromazine
- *Trans*-flupentixol

Antihypertensives
- Reserpine
- Propanolol

Antibiotics
- Erythromycin
- Gramicidin A

Steroids
- Corticosterone
- Dexamethasone
- Aldosterone
- Cortisol

Pesticides
- Methylparathion
- Endosulfan
- Cypermethrin
- Fenvalerate

Natural products
- Curcuminoids
- Colchicine

Antialcoholism drug
- Disulfiram

the uptake of these dyes into cells by flow cytometry, and its inhibition by other substrates and modulators, is an excellent indicator of transporter function. Fluorescent transport assays can also be used to distinguish between drug pumps. For example, calcein is a specific substrate for MRP1 and is not transported by either Pgp or ABCG2. Calcein-AM, on the other hand, is an excellent substrate for Pgp and forms the basis for commercially available kits for screening Pgp substrates.

MDR modulators

Drug resistance resulting from ABC multidrug transporters can be prevented by another group of compounds, known variously as MDR modulators, reversers, inhibitors or chemosensitizers [25]. Cells do not display resistance to modulators. In intact cells, the observation is that a modulator, when combined with a drug to which cells are resistant, will restore its cytotoxicity by shifting the LD_{50} to a much lower value. Modulators show the same diversity of chemical structure as substrates, and appear to act in several different ways. The mechanism of action of modulators has been explored in detail for Pgp. Some Pgp modulators compete with transport substrates for the drug-binding pocket of the transporter. Many

Box 2. Clinically relevant drugs and other compounds that interact with MRP1 (ABCC1).

Anticancer drugs
- *Vinca* alkaloids (vinblastine and vincristine)
- Anthracyclines (doxorubicin and daunorubicin)
- Epipodophyllotoxins (etoposide and teniposide)
- Camptothecins (topotecan and irinotecan)
- Methotrexate

Metalloids
- Sodium arsenate
- Sodium arsenite
- Potassium antimonite

Peptides
- Glutathione (GSH, GSSG)

Glutathione conjugates
- Leukotrienes C_4, D_4 and E_4
- Prostaglandin A_2-SG
- Hydroxynonenal-SG
- Aflatoxin B_1-epoxide-SG
- Melphalan-SG
- Cyclophosphamide-SG
- Doxorubicin-SG

Sulfate conjugates
- Estrone-3-sulfate
- Dehydroepiandrosterone-3-sulfate
- Sulfatolithocholyl taurine

Pesticides
- Fenitrothion
- Methoxychlor

Toxins
- Aflatoxin B_1

Glucuronide conjugates
- Glucuronosylbilirubin
- Estradiol-17-β-D-glucuronide
- Etoposide-glucuronide
- NS-38-glucuronide

HIV protease inhibitors
- Ritonavir
- Saquinavir

Tyrosine kinase inhibitors
- Imatinib mesylate
- Gefitinib

Fluorescent compounds
- Calcein
- Fluo-3
- BCECF

Antibiotics
- Difloxacin
- Grepafloxicin

Folates
- Folic acid
- L-leucovorin

Natural products
- Curcuminoids

of these compounds are transported by the efflux pump (e.g., cyclosporin A or verapamil) and can be viewed as competitive inhibitors. On the other hand, several hydrophobic steroid modulators are not transported by Pgp, but bind to a high-affinity site close to the ATP-binding site [26]. The high-affinity modulator XR9576 appears to interact with Pgp at a location distinct from the site of interaction of transport substrates, which may serve a modulatory function [27]. The modulator disulfiram (used to treat alcoholism) interacts with Pgp and MRP1 in a unique manner, binding to the drug-binding pocket and also modifying cysteine residues at the catalytic site [28]. Overall, the different mechanisms by which modulators exert their action at the molecular level are not well understood, making the rational design of new templates a challenging task.

Role of ABC multidrug transporters in normal physiology

The ABC drug efflux pumps Pgp, MRP1 and ABCG2 play a central role in protecting organisms from the toxicity of a variety of endogenous and exogenous molecules [29]. The tissues or organs that are protected depend on the specific pattern of expression and the activity of each protein in normal tissues. These transporters are, thus major contributors to the absorption, distribution and excretion of clinically administered drugs. Studies on knockout mice lacking each of these efflux pumps have confirmed these ideas [30–33].

Pgp is expressed at high levels in the apical membranes of epithelial cells lining the colon, small intestine, pancreatic and bile ductules, and the kidney proximal tubule. It is also found in the endothelial cells lining capillaries in the brain, testis and inner ear. Pgp-knockout animals display a disrupted blood–brain barrier, and can be up to 100-fold more sensitive to many drugs, which often show neurotoxicity not seen in wild-type animals [34]. The pregnant endometrium, the placenta and the adrenal gland also express high levels of Pgp. The location of Pgp suggests that its primary physiological role is to protect sensitive organs and the fetus from toxic xenobiotics [35]. In the intestine, Pgp extrudes many drugs into the lumen, thus reducing their absorption and oral bioavailability. It may export endogenous steroid hormones from the adrenal gland. The presence of Pgp in hematopoietic progenitor cells protects the bone marrow from the toxicity of chemotherapeutic drugs [36].

Box 3. Clinically relevant drugs and other compounds that interact with ABCG2.

Anticancer drugs
- Mitoxantrone
- Bisantrene (R482T mutant form)
- Epipodophyllotoxins (etoposide and teniposide)
- Camptothecins (topotecan and irinotecan)
- Flavopiridol
- Anthracyclines (doxorubicin and daunorubicin; R482T mutant form)

Antifolates
- Methotrexate

Porphyrins
- Pheophorbide a
- Protoporphyrin IX
- Hematoporphyrin

Tyrosine kinase inhibitors
- Imatinib mesylate
- Gefitinib

Flavonoids
- Genestein
- Quercetin

Carcinogens
- Aflatoxin B
- PhiP

Fungal toxins
- Fumitremorgin C
- Ko143

Drug & metabolite conjugates
- Acetominaphen sulfate
- Estrone-3-sulfate
- Dehydroepiandrosterone sulfate
- Estradiol-17-β-D-glucuronide
- Dinitrophenyl-S-glutathione

HMG CoA reductase inhibitors
- Rosuvastatin
- Pravastatin
- Cerivastatin

Antihypertensives
- Reserpine

Antibiotics
- Ciprofloxacin
- Norfloxacin

Fluorescent compounds
- Hoechst 33342
- BODIPY-prazosin
- Rhodamine 123 (R482T/G mutants)

Antiviral drugs
- Zidovudine
- Lamivudine

Natural products
- Curcuminoids

ABCG2 is expressed in a variety of normal tissues, including intestine, kidney and placenta, as well as brain endothelial cells and hematopoietic stem cells. Its expression is strongly induced in the mammary gland during pregnancy and lactation. Similarly to Pgp, it is assumed to function in protecting tissues from toxicants, and it likely plays a role in intestinal absorption, brain penetration and transplacental passage of drugs. In the mouse, ABCG2 actively concentrates chemotherapeutic drugs and a major dietary carcinogen into milk [37]. A recent report indicates that ABCG2 is responsible for secretion of riboflavin (vitamin B_2) into milk [38], indicating that it also plays an important physiological role. *ABCG2*-knockout mice display the disease protoporphyria, which results from uptake from the gut of the chlorophyll breakdown product, pheophorbide a, a normal constituent of food [39]. The absorption of this compound from the diet is normally limited by ABCG2. The transporter is also expressed in certain stem cells, where it acts as a marker of pluripotent stem cells (the side population). In these cells, ABCG2 appears to interact with heme and prevent accumulation of porphyrins, enhancing cell survival under hypoxic conditions [40].

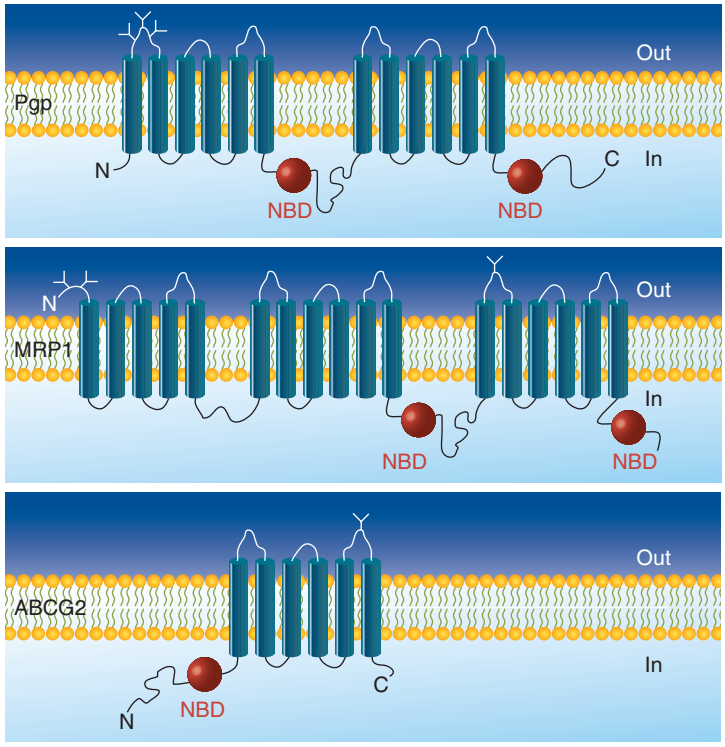
In contrast to Pgp and ABCG2, MRP1 is expressed at the basolateral membrane of polarized epithelial cells. It protects tissues such as the bone marrow, kidney collecting tubules, and oropharyngeal and intestinal mucosa, from toxicants, and is also involved in drug clearance from the cerebrospinal fluid, testicular tubules and peritoneum [29]. MRP1 plays a central role in glutathione homeostasis *in vivo*, and exports LTC_4 from mast cells. It may also be involved in protecting cells from the toxicity of bilirubin.

ATP binding & hydrolysis

The NBDs of all ABC proteins contain three highly conserved sequence motifs that play a critical role in ATP binding and hydrolysis; the Walker A and Walker B motifs, found in many proteins that bind ATP or GTP, and a signature C motif unique to the ABC superfamily. Site-directed mutagenesis approaches have revealed the importance of these three regions to catalytic function [41]. Structural studies of isolated NBD subunits from several ABC proteins have yielded useful information on the catalytic cycle. In the structures of BtuCD [42] and Rad50cd [43] (catalytic domains of a DNA-repair enzyme) the two NBDs are in close contact to form a dimeric structure. Each ATP-binding site is formed from

the Walker A and B motifs of one NBD subunit, and the LSGGQ signature C motif of the partner NBD subunit. Two molecules of ATP are bound in these sites at the dimer interface. This 'sandwich dimer' structure has also been observed for the NBD subunit of the bacterial ABC proteins MJ0796 and HlyB (Figure 2). These NBDs form a stable dimer in the presence of ATP when the catalytic activity is inactivated by mutation [44,45]. It seems likely that this NBD dimerization process plays a critical role in the catalytic cycle of all ABC proteins.

Figure 1. Predicted membrane topology of the drug-efflux transporters of the ABC superfamily.



Pgp is a full length transporter with 12 transmembrane (TM) segments and two cytoplasmic NBDs, which arose from a gene duplication. Both the N- and C-termini are cytoplasmic, and the first extracellular loop contains three glycosylation sites [185]. The topology of MRP1 is similar to that of Pgp, but it possesses an additional N-terminal domain with five TM segments, which is of unknown function. The extracellular N-terminus carries two oligosaccharide chains, while the C-terminus is cytoplasmic, with an additional glycosylation site on an extracellular loop [186]. ABCG2 is a 'half-transporter' comprising six TM segments and a cytoplasmic NBD that is located on the N-terminal side of the TM domain, in contrast with Pgp and MRP1. A single glycosylation site is located on the third extracellular loop [187]. ABCG2 is presumed to homodimerize to form a functional transporter.
MRP: Multidrug-resistance-associated protein; NBD: Nucleotide-binding domain; Pgp: P-glycoprotein.

All the ABC drug-efflux pumps display constitutive ATPase activity, which appears to be uncoupled from transport, and takes place in the absence of substrates. In the case of Pgp, basal ATPase activity is as high as 3–5 μmol/min per mg for the purified protein, depending on the presence of detergent, lipids and drugs [46,47]. The K_m for ATP is high (0.2–0.5 mM) [48,49], indicating that Pgp has a relatively low affinity for nucleotides. ABCG2 appears to have a similarly high K_m value for ATP, while MRP1 has a considerably lower K_m of approximately 100 μM [50].

The basal ATPase activity of Pgp is modulated by drug substrates and modulators in a complex manner. Many substrates show a biphasic pattern, stimulating activity at low concentrations and inhibiting at higher concentrations, whereas others show only stimulation or inhibition (e.g., [51–53]). The presence of different detergents and lipids also affects the drug interaction patterns [54,55] There is currently no satisfactory explanation for these observations, although it has been suggested that the biphasic pattern might arise from the presence of two drug-binding sites, a high-affinity stimulatory site and a low-affinity inhibitory site [56]. The increase in ATPase activity (and presumably transport) on addition of drug was correlated with the predicted degree of hydrogen bonding of substrate in the drug-binding pocket [57]. Substrates with extensive H-bonding showed low ATPase stimulation and low transport rates, whereas those with low levels of H-bonding expressed high ATPase stimulation and correspondingly high transport rates. The stoichiometry of ATP hydrolysis relative to substrate transport is a controversial issue but, overall, the data suggest that 1–2 ATP molecules are hydrolyzed for each drug molecule transported by Pgp.

The use of the ATPase inhibitor, ortho-vanadate (V_i, an analog of P_i), has led to some important mechanistic insights. The ATPase activity of Pgp is rapidly inhibited by the addition of V_i in the presence of ATP. V_i is trapped after a single catalytic turnover in only one NBD as the complex $ADP{\cdot}V_i{\cdot}Mg^{2+}$, which suggests that both catalytic sites must be functional for ATP hydrolysis to take place. Pgp was therefore proposed to operate by a mechanism in which only one catalytic site is in the transition state conformation at any time, and the two sites alternate in catalysis [58]. The V_i-trapped complex is very stable, and is believed to resemble the catalytic transition state structurally. MRP1 and ABCG2 are also

inhibited by V_i, suggesting that the proposed mechanism may be common to these ABC proteins as well. However, the two NBDs of MRP1 are clearly not functionally or structurally equivalent, and V_i trapping occurs predominantly at the C-terminal NBD [59].

Nucleotide binding to ABC drug-efflux pumps has been investigated using photoaffinity labeling with azido-nucleotide analogs and, in the case of Pgp, fluorescence and electron paramagnetic resonance (EPR) spectroscopy. The latter two techniques allowed estimation of the affinity and stoichiometry of binding [60,61]. The K_d values for ATP binding to Pgp are in the range of 0.2 to 0.5 mM, similar to the K_m for ATP hydrolysis. Both NBDs are occupied with ATP in the native protein, and one ATP molecule can bind to the vacant NBD in the V_i-trapped state [60].

Drug binding

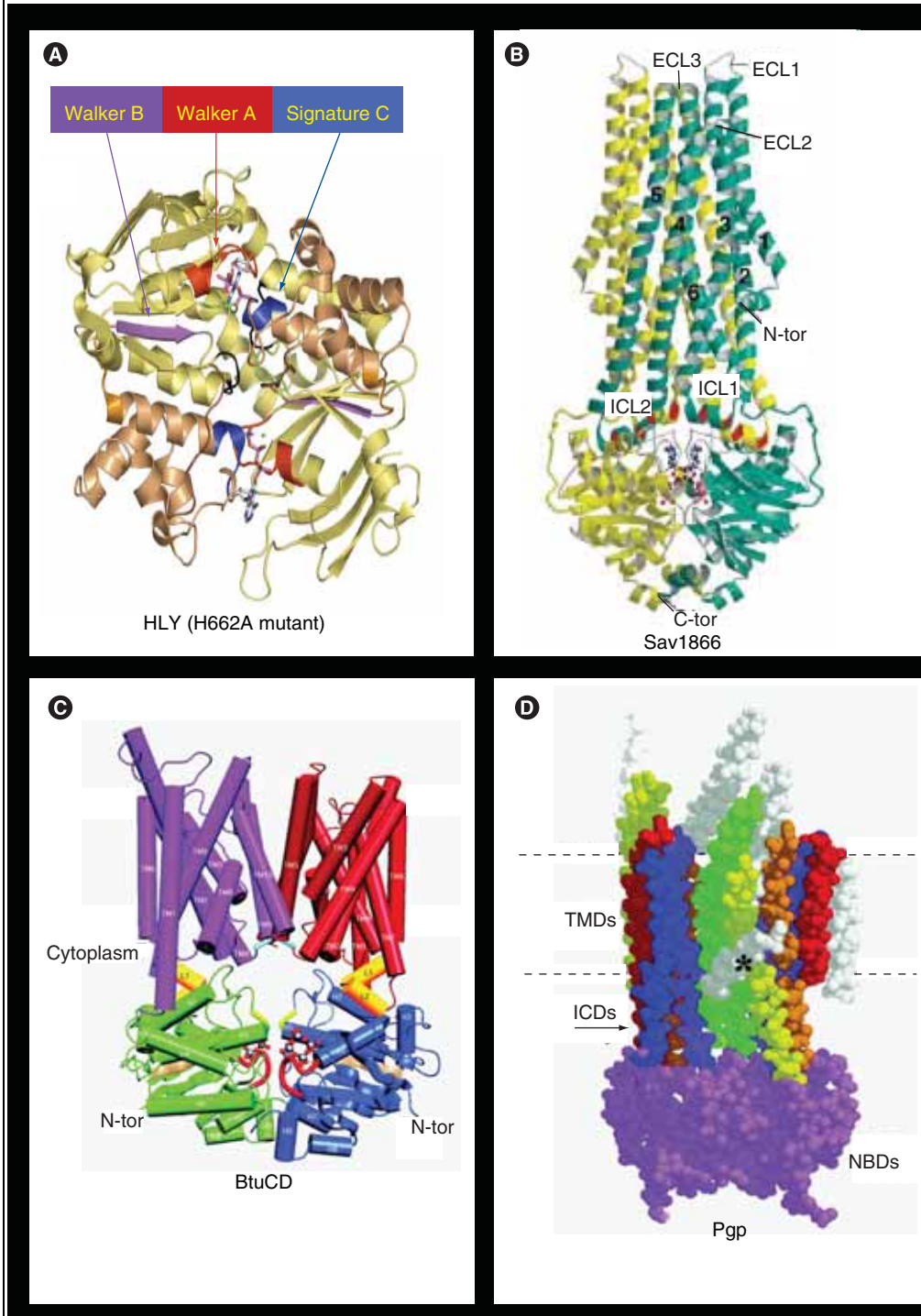
Biochemical studies have revealed the membrane topology of the mammalian ABC drug pumps. Pgp is a single polypeptide that arose from an internal duplication, and comprises 12 TM segments and two NBDs (Figure 1). ABCG2 is a half-transporter with six transmembrane (TM) segments and a single NBD, which is assumed to homodimerize to form the transport-competent complex [9]. In Pgp, the NBDs are C-terminal to the TM regions in the primary sequence, whereas in ABCG2 their order is reversed. MRP1 resembles Pgp, but has an extra N-terminal domain, TMD_0, consisting of five TM segments. The functional role of this third membrane-spanning domain is currently unclear. It is not required for transport or proper trafficking to the plasma membrane in polarized cells [62], however, if the C-terminus of MRP1 is mutated, the TMD_0 is essential for normal targeting [63]. MRP1 appears to exist as a dimer in the membrane, and it has been suggested that TMD_0 and the associated linker region may mediate dimerization [64].

There are currently no high resolution structures available for any eukaryotic ABC protein. The best existing information is a medium resolution structure of Pgp determined by cryo-electron microscopy (Figure 2D) [65]. This structure shows that Pgp has 12 TM helices and two closely apposed NBDs, supporting the proposed topology. Fluorescence spectroscopic and biochemical cross linking studies are also compatible with this structure [66–68]. Electron microscopic structures have been reported for MRP1 [69] and ABCG2 [70], but these are of low resolution and have added little to our understanding of how these proteins function.

The nature and location of the drug-binding site(s) have been extensively investigated for Pgp and MRP1, whereas less is known for ABCG2. The Pgp drug-binding pocket is made up by the TM helices of the protein [71], and is located within the cytoplasmic membrane leaflet. Several drug binding subsites or minipockets appear to exist that interact with each other sterically or allosterically in a complex fashion, so that transport of one drug is either stimulated or inhibited by binding of a second drug [72,73]. The drug-binding pocket within the protein is shaped like a funnel that is narrower at the cytoplasmic side, and it appears large and flexible enough to accommodate two drug molecules simultaneously [74,75]. Substrates may enter this region through 'gates' formed by the cytoplasmic ends of TM helices 2/11 and 5/8, which are close together [76]. The amino acids lining the binding pocket hold the drug in place via Van der Waal's forces, hydrophobic interactions and hydrogen bonding. The flexible binding site allows structurally unrelated drugs to establish interactions with different subset of residues within the binding pocket, so that binding takes place by an induced-fit type of mechanism [77]. In this way, drug binding to Pgp resembles the process in the bacterial multidrug-binding transcriptional regulators, such as QacR and BmrR. For these soluble proteins, the principles of multidrug binding have been well-established, due to the existence of crystal structures showing how different drugs bind [78]. The drug-binding pocket of Pgp has been suggested to be accessible to the external aqueous solution [79], although fluorescence measurements indicate that the bound drug molecule is in a relatively hydrophobic environment [80].

Substrate binding to the ABC drug efflux pumps has been characterized using photoactivatable drug analogs, radiolabeled drug-binding studies, and fluorescence-quenching approaches. The measured range of K_d values for drug binding to Pgp covers over four orders of magnitude [81], demonstrating that the transporter can effectively discriminate between compounds, and is polyspecific rather than nonspecific. Attempts to generate a quantitative structure-activity relationship (QSAR) for Pgp have been challenging. The best description of a Pgp substrate involves a set of structural elements, including two–three hydrogen bond acceptors and hydrophobic groups,

Figure 2. High-resolution x-ray crystal structures.



(A) Nucleotide sandwich dimer of the ATP-binding subunit, HlyB (catalytically inactive H662A mutant; taken from [45], reprinted with permission from Elsevier Limited).
(B) The bacterial multidrug efflux pump Sav1866 (taken from [92], reprinted with permission).
(C) The bacterial vitamin B_{12} importer BtuCD (taken from [42], reprinted with permission from AAAS).
(D) The medium resolution cryoelectron microscopic structure of Pgp (taken from [65], reprinted with permission).
ECL: Extracellular loop; ICD: Intracellular domain; ICL: Intracellular loop; NBD: Nucleotide-binding domain; Pgp: P-glycoprotein; TMD: Transmembrane domain.

arranged in a fixed spatial orientation [82–84]. Substrates with diverse structures are envisaged as positioning themselves differently among these elements so as to maximize their interactions. Binding strength would depend on the number of interaction points and the strength of the hydrogen bonds. The TM helices of Pgp contain a large number of amino acid side chains that could serve as hydrogen bond donors to aid in binding drug molecules. Aromatic amino acids, particularly Trp residues, may play an important role in stacking with substrates that contain aromatic rings [85], since their fluorescence properties are sensitive to drug binding [86]. The drug-binding pocket of ABCG2 may function in a similar way to that of Pgp. Radioligand binding studies showed that there appeared to be at least two symmetric substrate binding sites within the protein, with overlapping specificity [87].

The drug-binding pocket of MRP1 may be bipartite in nature, since it is able to accommodate both a hydrophobic moiety and a negatively charged glutathione group. The core region of the protein, which contains the substrate-binding region, has been studied extensively using site-directed mutagenesis and crosslinking with photoactivatable substrate analogs [88]. In addition, molecular models of MRP1 have been developed to integrate this data into a 3D structure [89]. The TM helices 10, 11, 16 and 17 appear to be important in binding transport substrates, with lesser contributions from other TM regions and cytoplasmic loops. A 'basket' of aromatic residues located close to the cytoplasm-membrane interface of MRP1 has been proposed to play a central role in the initial interaction of drugs with the protein [89]. TM helix 6 may be involved in recognition of glutathione and its conjugates.

Transport mechanism

There has been increasing success in crystallizing integral membrane proteins over the past few years, and the high-resolution structures of several intact bacterial ABC proteins have been determined, including BtuCD [42], HI1470/1471 [90], $ModB_2C_2$ [91] and Sav1866 (Figure 2) [92]. BtuCD, HI1470/1 and $ModB_2C_2$ are metal chelate importers, and like all bacterial ABC importers, they are associated with a periplasmic binding protein that delivers the substrate to the membrane-bound complex. There is now a structural basis for understanding how these complexes work to transport substrate from the periplasmic space to the cytosol [91,93]. However, this class of proteins sheds little light on the structure or possible mechanism of action of mammalian ABC drug-efflux pumps. Three high-resolution structures of the bacterial lipid A transporter, MsbA [94], were recently withdrawn [95]. The homology of MsbA to Pgp had made these structures attractive to other researchers in the field, and they were widely used as the basis for homology models and molecular dynamics simulations [96,97]. At this time, it is not clear how valid these models and simulations are. Sav1866 is a putative bacterial multidrug exporter with 12 TM helices, and thus more closely resembles mammalian drug efflux pumps. It remains to be seen how useful this structure will be in its application to the mammalian multidrug transporters.

Drug transport involves two interconnected cycles [98]. First there is the catalytic cycle of ATP hydrolysis, which drives transport. Second, there is the substrate transport cycle, whereby a drug molecule is moved from the cytoplasmic side to the extracellular side of the membrane. Details of the catalytic and transport cycles, and how they are coupled, remain enigmatic. In many studies, Pgp has appeared to behave as a symmetrical molecule, with two equivalent NBDs (although this is controversial, see [99]), and it is likely that the ABCG2 homodimer also functions symmetrically. On the other hand, there is considerable evidence that the NBDs of the ABCC subfamily are not structurally or functionally equivalent, and the two NBDs of MRP1 may each perform a different role in the catalysis and transport cycle [59].

The catalytic cycle involves low affinity binding of ATP to both NBDs, which induces formation of a putative nucleotide sandwich dimer. In the case of Pgp, this may involve bringing the NBDs into very close apposition to form two composite catalytic sites at the interface of the domains. One molecule of ATP appears to become bound very tightly (occluded) at this stage [100], and is probably committed to enter the catalytic transition state, thus being hydrolyzed to ADP and P_i. After ATP hydrolysis, which appears to be the rate-limiting step for ABC proteins, the alternating sites mechanism [58] proposes that ADP and P_i are released from one NBD, which then reloads with ATP. The second round of ATP hydrolysis then takes place at the catalytic site of the partner NBD. It is not known how this cooperation between the two active sites is achieved. An alternative, processive clamp mechanism has been proposed [101] in which both ATP molecules are hydrolyzed in succession before release of

ADP and P_i and reloading with two more ATP molecules. Mechanistic schemes have been proposed in which either one or two molecules of ATP are hydrolyzed for each drug molecule transported [58,102].

Drug transport by the MDR efflux pumps starts with entry of the substrate into the binding pocket on the cytoplasmic side, followed by protein conformational changes (driven by ATP binding or hydrolysis), and release of the drug into either the extracellular aqueous space or the opposing membrane leaflet. The drug is thought to initially interact with a high-affinity binding site, and then be moved to a low-affinity site for release. The coupling between drug transport and ATP hydrolysis involves communication between the two domains, linked by conformational changes, which have been demonstrated for Pgp by fluorescence spectroscopy [103]. There is an ongoing debate about whether the energy for drug transport by Pgp is provided by ATP hydrolysis [102] or ATP binding (the ATP switch model [104]).

Role of the lipid bilayer in drug binding & efflux

The lipid bilayer plays an important part in the efflux function of Pgp (and probably ABCG2), whose substrates are typically hydrophobic and lipid-soluble. The idea that the transporter acts as a 'vacuum cleaner' for hydrophobic molecules present within the membrane was suggested [105] and is now widely accepted. In intact cells, drugs entering the cell from the extracellular side are intercepted at the plasma membrane and transported to the exterior without entering the cytosol (Figure 3A). The binding process comprises two steps; partitioning of drug from water into the membrane, and subsequent transfer of drug from the lipid to the binding pocket of the protein. Because the membrane concentrates drugs up to 1000-fold for presentation to the transporter, the intrinsic affinity of the transporter for its substrates may be quite low. This was confirmed by a thermodynamic analysis of the drug-binding process within a lipid bilayer [106]. The free energy of binding of a drug to Pgp within the lipid milieu correlates well with the cross-sectional area of the molecule, which in turn likely reflects the number of favorable interactions that the drug can make with the protein TM helices [106]. The physical properties of the membrane modulate drug binding and transport by Pgp reconstituted into lipid bilayer vesicles. Drugs with high lipid–water partition coefficients demonstrated higher apparent binding affinities, in keeping with the vacuum cleaner model (Figure 3B) [107]. The initial rate of substrate transport was also dependent on the lipid fluidity, and reached a maximum at the lipid melting temperature [108].

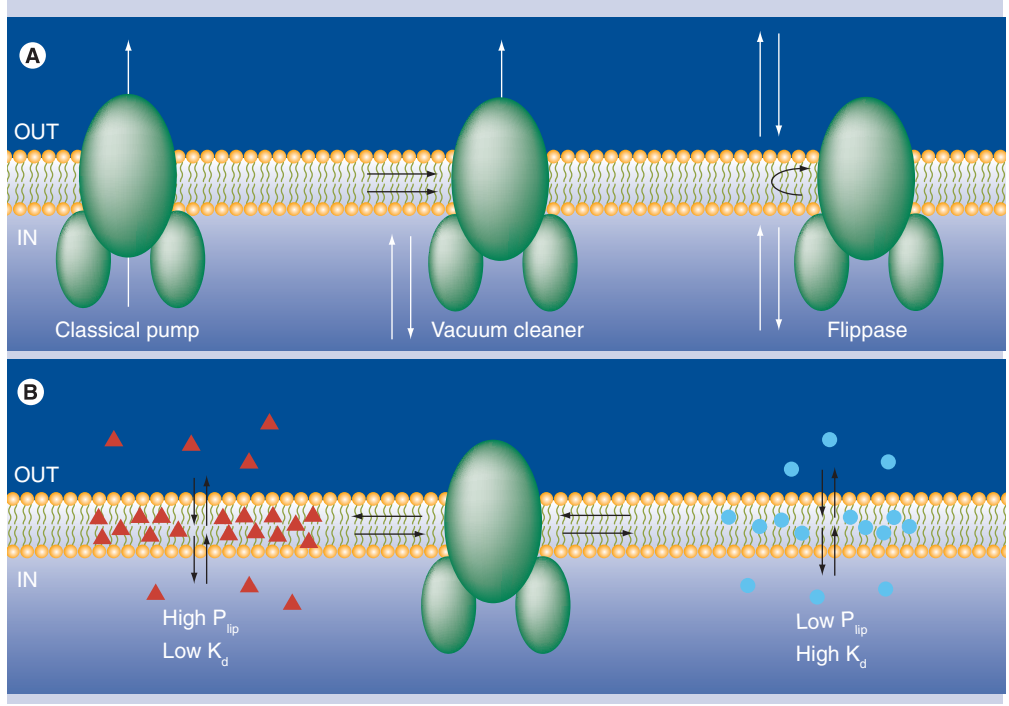
Drugs approaching the extracellular side of the plasma membrane will rapidly partition into the outer leaflet, then 'flip-flop' into the inner leaflet, a process that is known to be slow for many Pgp substrates [109]. Pgp may expel drugs from the membrane into the aqueous phase directly, or it may 'flip' them from the inner to the outer leaflet of the bilayer, from which they can rapidly partition into the aqueous phase (Figure 3A). Reconstituted Pgp is able to flip fluorescent analogs of both phospholipids and simple glycosphingolipids [110], and interacts with many lipid-based drugs [14], indicating that it may also be a lipid transporter. The closely related ABCB4 protein exports phosphatidylcholine into the bile, likely by a flippase mechanism. Given the resemblance in function and substrate profiles between Pgp and ABCG2, it is possible that the latter operates by a similar mode of action.

Modulators that inhibit Pgp transport may function within the milieu of the bilayer. It has been proposed that the feature which distinguishes this group of compounds is their high rate of spontaneous movement across the membrane [109]. Substrates are suggested to cross membranes at a low rate. After they have been effluxed, they will rapidly repartition into the outer leaflet, cross slowly to the inner leaflet and interact with Pgp once again, but the transporter will be able to keep pace and maintain a substrate concentration gradient. Modulators, on the other hand, appear to cross membranes very rapidly, faster than the rate of Pgp-mediated transport. Thus, the protein engages in a futile cycle of modulator transport, but cannot either generate a modulator gradient or transport other substrates. Therfore, although modulators inhibit transport of other drugs, it is difficult to measure the rate at which they are themselves transported.

ABC drug-efflux pumps in tumors

Our understanding of the involvement of ABC transport proteins in drug-resistant cancers is still evolving. High expression of Pgp has been observed prior to chemotherapy treatment in many different tumor types, including kidney, colon, liver, breast and ovarian cancers. In

Figure 3. Classical pump, vacuum cleaner and flippase models of Pgp action.



(A) Pumps (e.g., lactose permease) transport polar substrates from one side of the membrane to the other, through a hydrophilic path formed by the transmembrane (TM) regions of the protein. In the vacuum cleaner model, drugs partition into the lipid bilayer, interact with P-glycoprotein (Pgp) within the membrane, and are subsequently effluxed extracellularly. In the flippase model, substrates partition into the membrane, interact with the drug-binding pocket in Pgp (located within the cytoplasmic leaflet) and are then translocated, or flipped, to the outer membrane leaflet. **(B)** The effect of membrane partitioning on drug binding to Pgp. The binding affinity of Pgp for a particular substrate or modulator (K_d) is related to the P_{lip}. A drug with a high value of P_{lip} (left side of the figure) will accumulate to a high concentration within the membrane. This will favor binding to Pgp and result in a low apparent K_d. In contrast, a drug with a low value of P_{lip} (right side of the figure), will have a lower membrane concentration, and a high apparent K_d; P_{lip}: Lipid–water partition coefficient of the drug.

hematological malignancies, such as leukemias, lymphomas and multiple myelomas, the low levels of Pgp expression observed initially are markedly increased after chemotherapy treatment and relapse. For acute myelogenous leukemia (AML), numerous studies have found a very good correlation between Pgp expression levels and poor prognosis and response to chemotherapy in adults, but not children [111,112]. Pgp expression also appears to be an adverse prognostic factor in adult (but not pediatric) acute lymphoblastic leukemia (ALL) [112].

Since its discovery in 1998, high levels of ABCG2 expression have been found on a variety of drug-resistant cell lines that do not express either Pgp or MRP1. Since then, ABCG2 expression has been reported in many solid tumors [113], especially those from the GI tract, endometrium, lung and melanoma. Some studies have reported high levels of ABCG2 in AML and ALL samples, which were associated with poor prognosis and response to clinical treatment, while others have contradicted these findings [114]. However, a case has been made for the likely involvement of ABCG2 in resistance of both adult and childhood AML to anticancer drugs [112].

Less is known about the involvement of MRP1 in tumors, and its role in drug resistance remains controversial. MRP1 is expressed in many different tumor types, including solid tumors (non-small-cell lung cancer, gastrointestinal carcinoma, melanoma, neuroblastoma and cancers of the breast, ovary and prostate), and hematological malignancies including leukemias [115]. Some studies have linked the presence of MRP1 to poor treatment outcome, but no comprehensive picture of its role in clinical MDR has emerged as yet.

Modulation of MDR efflux pumps in cancer treatment

Large numbers of modulators have been identified for Pgp over the past 25 years [25]. In contrast, only a few modulators have been described for MRP1 [116], including VX-710 (biricodar) MK571 (a leukotriene D_4-receptor antagonist), flavonoids, raloxifene analogs, isoxazole-based compounds and glutathione derivatives. ABCG2 inhibitors have only recently been investigated, and include VX-710, GF120918 (elacridar), XR9576 (tariquidar), fumitremorgin C (a mycotoxin) and its derivative Ko143, pantoprazole, flavonoids, estrogens and antiestrogens [117]. The most sought-after characteristic of modulators has been their ability to reverse resistance to commonly used anticancer drugs, and many clinical trials targeting Pgp have been carried out. However, translation of basic biochemical knowledge of the ABC efflux pumps to clinical applications at the bedside has proved difficult. Very few of the hundreds of modulators identified *in vitro* are suitable for clinical application in cancer treatment (Box 4). The development of modulators that are effective against MDR tumors, yet nontoxic, has been a major challenge over the past two decades. For example, fumitremorgin C, which is a highly specific ABCG2 inhibitor, is too neurotoxic for clinical use.

Box 4. Clinically relevant modulators that interact with Pgp, MRP1 and ABCG2.

P-glycoprotein: first generation

- Verapamil
- Cyclosporin A
- Tamoxifen

Second generation

- PSC833 (valspodar)
- VX-710 (biricodar)

Third generation

- LY335979 (zosuquidar)
- XR9576 (tariquidar)
- GF120918 (elacridar)
- OC144–093 (ontogen)

MRP1

- VX-710 (biricodar)

ABCG2

- GF120918 (elacridar)
- Ko143
- Pantoprazole
- XR9576 (tariquidar)
- VX-710 (biricodar)
- Gefitinib?
- Imatinib?
- Quercetin?

The extent of involvement of ABC efflux pumps in anticancer drug resistance, and whether modulation can result in increased patient survival, remains controversial. The first generation of Pgp modulators used clinically (verapamil and cyclosporine A) were generally drugs already in use for treatment of other medical conditions, and they suffered from the dual problems of high toxicity and low efficacy at tolerable doses. Second-generation modulators (PSC833 and VX-710) showed improved efficacy at low doses, but serious adverse pharmacokinetic interactions were often noted in cases where both the treatment drug and the modulator were substrates for cytochrome P450 3A. Reduced clearance of the anticancer drug led to increased toxicity to the patient. Third-generation modulators, including LY335979 (zosuquidar), XR9576 and OC144–09 (ontogen), have low toxicity and show both increased selectivity and high potency against Pgp. There is hope that these new agents will prove more successful in clinical trials of Pgp modulation in cancer, which have so far been disappointing [118].

Several ABCG2 modulators that could be used in patients have been identified in the past few years (Box 4) [114], however, none has yet been used in a clinical trial. Given the fact that many of the compounds identified as ABCG2 inhibitors also act on Pgp (e.g., GF120918), the possibility of using dual Pgp–ABCG2 modulators clinically appears to be a realistic goal. Some multifunctional modulators have been identified that inhibit the activity of all three efflux pumps, for example, VX-710 appears to block Pgp, MRP1 and ABCG2 [119].

Role of ABC multidrug transporters in drug therapy

Many drugs commonly used in clinical therapy are transport substrates for Pgp, MRP1 and ABCG2 (Boxes 1, 2 & 3). The ABC proteins thus play an important role in absorption and disposition of these drugs *in vivo*. Both ABCG2 and Pgp can limit the uptake of many drugs in the intestine. The presence of these efflux pumps is a serious problem in drug discovery, since many new drug candidates may not be able to cross the intestinal barrier *in vivo*, making them clinically useless. Drug discovery screening at many pharmaceutical companies now includes testing for interactions with ABC transporters. The

presence of efflux pumps in the endothelial cells of the brain capillaries also has a far-reaching impact on pharmacotherapy of brain diseases, including cancer, AIDS, Parkinson's disease, epilepsy and schizophrenia [120,121]. Several ABC transporters (the most important are Pgp and ABCG2) are present in the luminal membrane of endothelial cells, where they immediately pump drugs back into the blood, thus greatly reducing their accumulation in brain tissue. By contrast, MRP1 is present in the choroid plexus epithelium, and contributes to the blood–cerebrospinal fluid (CSF) drug-permeability barrier by preventing transfer of drugs into the CSF.

Studies using knockout mice have demonstrated that access of many drugs to the brain can be increased five- to 100-fold in the absence of Pgp and ABCG2 [122,123]. In *MRP1*-knockout mice, levels of etoposide in the CSF are increased tenfold compared with wild-type mice [124]. These observations have generated much interest in using Pgp and ABCG2 modulators in conjunction with therapeutic drugs to enhance their oral bioavailability and delivery to the brain [125]. This approach is known to be successful in a mouse model, and future application to humans may improve the therapeutic effectiveness of drugs targeted to the central nervous system.

Polymorphisms in ABC drug-efflux pumps

In recent years, there has been considerable interest in how polymorphisms in ABC efflux pumps may affect drug therapy in these individuals [126]. Many SNPs in Pgp, MRP1 and ABCG2 have been identified in different human populations (see below for details), and are thought to play a major role in the variations in drug responses observed in different individuals and ethnic groups. A point mutation that occurs in at least 1% of the population is considered to be an SNP, which may be nonsynonymous (giving a change in the coding sequence) or synonymous (silent). SNPs may result in differences in both protein expression level and transport function, which are in turn expected to affect drug absorption, plasma concentration, distribution, and elimination. More extensive changes in the genome sequence encoding these proteins are also possible. For example, some dog breeds (e.g., Collie) lack a Pgp arising from a frameshift mutation and are hypersensitive to certain drugs. Despite the widespread clinical use of drugs that are substrates for Pgp, ABCG2 and MRP1, there have been no reports of human null alleles. Details of naturally occurring human ABC transporter polymorphisms are available in several databases [201–203].

Effect of polymorphisms & mutations on Pgp expression & function

Pgp variants carrying spontaneous mutations have been found in cultured cell lines; the first one to be identified was G195V, which caused increased resistance to colchicine, while resistance to several other drugs was unchanged [127]. Another cell line with a spontaneous deletion of F335 showed altered resistance to several substrates [128].

Genetic polymorphisms in *ABCB1* have been reported to change the mRNA expression, protein expression and function of Pgp [129]. The first SNP reported for human Pgp was the G2677T variant, which is a nonsynonymous SNP resulting in a change in the coding sequence, A893S. Over 50 SNPs and insertion/deletion polymorphisms in the *ABCB1* gene have been reported to date [126,129–133]. Distinct haplotypes exist [134], with considerable heterogeneity within various populations, although all ethnic groups appear to have the three most common haplotypes. One common haplotype includes the SNPs C1236T (exon 12, synonymous), G2677T (exon 21, nonsynonymous, A893S) and C3435T (exon 26, synonymous), and is found frequently in European Americans, whereas C1236C-G2677T-C3435C haplotype is common in Africans [135].

The synonymous C3435T polymorphism was reported to be associated with reduced Pgp mRNA expression in a few studies, but this was contradicted by others, and the overall consensus is that such an association is not significant [129]. Several other single polymorphisms in Pgp have also failed to show an association with levels of protein expression [129], leading to inconclusive results overall. More recent work examining protein expression levels has focussed on associations based on haplotypes, however, the results have again been inconclusive, so that an association between haplotype and Pgp mRNA or protein expression has not been demonstrated. Confounding factors in these studies include medications, diet, interindividual differences in drug metabolism and the presence of underlying disease.

Recent work has demonstrated that expression of C3435T results in Pgp that has a slightly different tertiary structure and altered interactions

with drugs and modulators, despite having the same amino acid sequence [136]. These functional changes were suggested to arise from altered folding kinetics during protein biosynthesis as a result of rare codon usage.

Expression in cultured mammalian cell lines of Pgps carrying SNPs identified in human populations has shown that many of these variants have little or no effect on either Pgp surface expression or transport function [137–139]. In recent reports, several non-synonymous polymorphisms expressed in mammalian and insect cells displayed modest changes in substrate specificity and drug-stimulated ATPase activity [139,140]. However, the nonsynonymous mutations of G2677T/A/C, which result in the amino acid changes A893S, A893T and A893P, gave changes in both substrate specificity and ATPase kinetic properties as measured with 41 different test compounds [139]. The extent of the observed functional change varied with the particular drug tested. The polymorphisms at amino acid 893 also show wide differences in their allele frequency in different ethnic groups. This location (which is within the second intracellular loop in the C-terminal half of Pgp) thus appears to be a hotspot for mutations, and these polymorphisms could indeed influence the disposition and therapeutic efficacy of various drugs administered clinically.

Influence of Pgp polymorphisms on drug therapy

Polymorphisms that affect Pgp expression or function would be predicted to be associated with changes in both the pharmacokinetics of administered drugs and the clinical outcome of drug therapy. Since Pgp is known to affect drug oral absorption, renal clearance and penetration into organs such as the brain, polymorphisms might alter all of these parameters. Hoffmeyer reported a twofold reduction in the levels of duodenal Pgp in C3435T subjects, which was associated with increased oral absorption and higher plasma levels of digoxin [141]. However, the majority of subsequent studies with other Pgp drug substrates (tacrolimus, fexofenadine and cyclosporine A) failed to confirm this [129]. Later work showed that the linked nonsynonymous SNP G2677T might be responsible for the observed association with reduced fexofenadine uptake, suggesting that this variant has increased activity *in vivo* [135]. The majority of attempts to demonstrate an association between *ABCB1* genotype/haplotype and pharmacokinetics for other drugs believed to be Pgp substrates have also been inconclusive [129]. Even the few studies that showed positive associations also reported contradictory data, and replication has been difficult.

If an association between *ABCB1* genotype and Pgp expression/activity is real, the clinical outcome of drug treatment with a Pgp substrate would also display this dependence. HIV-protease inhibitors are known to be Pgp substrates, and transport activity would be expected to reduce both oral uptake of these drugs, and their penetration into the brain. The G1199A polymorphism (S400N) affected the trans-epithelial transport of five HIV protease inhibitors [140]. An association appears to exist between certain Pgp variants and the outcome of treatment with antiretroviral therapy for AIDS. Patients with 3435TT phenotypes who initiated antiretroviral therapy showed better recovery of immune function after 6 months [142], while those with the 3435CC genotype tended to have earlier treatment failure due to high viral load [143]. A more rapid response to nelfinavir therapy was also noted in HIV-1-infected children with the 3435CT genotype compared to those with the CC genotype [144]. However, several studies reported no significant association of genotype with clinical outcome for this group of drugs [129].

A more well-documented link has been reported between epilepsy that is refractory to treatment with multiple drugs and the C3435T polymorphism in the *MDR1* gene [145]. Patients with drug-resistant epileptic seizures were more likely to show homozygosity for the CC genotype, a polymorphism that is associated with increased Pgp-transport function. These results suggest that drugs are less able to cross the blood–brain barrier in this patient group. However, these findings have not been confirmed by more recent studies [129,146–148].

Two common Pgp polymorphisms, G2677T/A and C3435T, may be involved in the differential response of patients to the cholesterol-lowering statins. The C3435T variant was associated with a lower response to atorastatin in female patients, and haplotype analysis identified a subgroup of individuals with a remarkable response to treatment that was not linked to a single polymorphism [149]. Response to treatment with fluvastatin was associated with a haplotype containing the G2677T/A allele [150]. Another group also reported a link between responses to several statins and the

polymorphism at position 2677, however, the association seen for the C3435T allele contradicted that of the first study [151].

The effects of Pgp polymorphisms on the outcome of anticancer drug treatment have been described for several different tumor types and treatment regimens [129]. Again, although some positive associations have been reported, no clear pattern has emerged to date.

Influence of Pgp polymorphisms on disease susceptibility

Since Pgp's play a central role in tissue defence against toxic substrates, it would be predicted that polymorphisms might alter susceptibility of individuals to disease states. In particular, Pgp may protect the GI tract from bacterial flora and their toxins, which is supported by the fact that *mdr1a*-knockout mice spontaneously develop colitis that progresses to dysplasia [152]. It is, therefore, not surprising that Pgp polymorphisms have been linked to inflammatory bowel disease, both Crohn's disease and ulcerative colitis [153–155]. Susceptibility to development of colon cancer is also increased by certain Pgp polymorphisms [156–158], and carriers of the 3435TT and 3435T genotype were at substantially increased risk in an under-50 patient population [157]. The same polymorphisms also appear to increase the risk of renal epithelial tumors [159].

A link to Parkinson's disease may also exist. Transport of the anti-Parkinson's drug, budipine, out of the brain is mediated by Pgp in mice [160], and susceptibility to the disease was reported to be associated with Pgp polymorphisms. The haplotype G2677T/C3435T appears to confer protection to Parkinson's disease in Chinese populations, although these findings have been disputed [146,161,162], and the association remains controversial.

Polymorphisms in *MRP1*

A number of *MRP1* gene polymorphisms have been identified and its haplotypes investigated [134,163–165]. The *MRP1* gene displays high haplotype diversity, and distinct differences between ethnic groups were noted [164]. The significance of these variants, and their potential role in drug delivery and disease susceptibility, has been explored by transfection of these proteins into mammalian cell lines, followed by characterization of their expression levels and transport function [163,166]. Most mutant MRP1 proteins were expressed at levels comparable to the wild-type, and only a few showed altered function. These results suggest that the majority of *MRP1* polymorphisms are unlikely to have effects on drug disposition. The G1299T polymorphism (exon 10) results in the change R433S in a cytoplasmic loop of MRP1, and was observed to increase doxorubicin resistance, but decrease transport of several organic anions [167]. The nonsynonymous change G128C (C43S) impaired the plasma membrane localization of the protein, and also decreased resistance to doxorubicin and sodium arsenite [168]. Very few *MRP1* polymorphisms have been associated with clinical disease or altered drug responses.

Mutations & polymorphisms in *ABCG2*

The oral bioavailability and clearance of drugs that are ABCG2 substrates is highly variable [169], suggesting that interindividual variations arising from polymorphisms might be important. Over 80 SNPs, missense, nonsense and frameshift mutations in the *ABCG2* gene have been identified in different ethnic groups [23,170], including V12M (N-terminal cytosolic region), Q141K (NBD) and Q126stop (in which no active protein is produced). Functional characterization of several of these polymorphisms has been carried out; some show increased transport activity, while others display reduced expression and/or function. In a study of six different SNP variants, the C421A polymorphism (nonsynonymous, Q141K) was expressed at lower levels, and the S441N variant had both lower expression and altered localization [171]. The Q141K mutation is located between the Walker A and signature motifs, so altered ATPase activity is possible. Compared with wild-type *ABCG2*, the Q141K variant displayed lower ATPase activity and lower mitoxantrone efflux when expressed in HEK-293 cells, whereas the V12M and D620N proteins showed little change [172]. Somewhat different results were reported by another group for the V12M and Q141K variants [173]. A recent study examined seven ABCG2 variants in detail, and found that cells expressing both V12M and Q141K had reduced resistance towards the drug SN-38 [174]. Several different studies that examined the expression level of the Q141K variant, both in human tissues and in transfected cell lines, have yielded contradictory results [175].

The frequency of the Q141K polymorphism varies considerably among different ethnic populations, being commonly found in China and Japan, and was found to be part of a

common haplotype [176,177]. Individuals carrying this polymorphism are predicted to show altered responses to anticancer agents and other drugs due to reduced efflux activity of ABCG2, and indeed, changes in the pharmacokinetics of several drugs were noted, with increased levels in the plasma [178–180]. The accumulation of the tyrosine kinase inhibitor gefitinib was higher in patients heterozygous at the C421A (Q141K) locus compared with those with a wild-type genotype, indicating that this polymorphism may indeed affect the outcome of anticancer drug treatment [181]. However, no significant effect of this polymorphism on irinotecan pharmacokinetics was observed [177]. The frequency of the CC genotype for the C421A polymorphism was significantly higher in renal cell carcinoma patients, suggesting that *ABCG2* may be a susceptibility gene for this cancer [182]. A recent study characterized the activity of 18 ABCG2 variants, and concluded that Q126stop, F208S, S248P, E334stop, S441N and F489L are defective in hematoporphyrin transport [170], which may increase the risk of disease in individuals carrying these polymorphisms.

A spontaneous mutation in *ABCG2* was identified in drug-selected cultured cell lines. Changes to R482 in the third TM segment resulted in a gain-of-function mutant that had altered substrate specificity [183]. Several other amino acid substitutions at R482 also led to large changes in drug transport and substrate specificity [184]. The R482T and R482G variants were able to efflux rhodamine 123 and doxorubicin, whereas the wild-type was not, however, all three forms of ABCG2 transported mitoxantrone. These results indicate that a single amino acid change at position 482 can alter the substrate specificity and drug-resistance phenotype, and suggest that this residue may play a critical role in ABCG2 function. However, to date, no SNP at this position has been found in human populations.

Future perspective

The central role played by ABC multidrug efflux pumps in protecting tissues from exogenous and endogenous toxins is now widely recognized. These related transporters play a central role in the uptake of drugs and delivery to their tissue targets, however, we have much to learn about the complex network of interactions between them. Substantial progress has recently been made in determining the high resolution structures of bacterial ABC proteins, however, structural information for the mammalian family members is very sparse. Much more structural and biochemical information will be necessary for a detailed understanding of the catalytic cycle and drug-transport mechanism of the ABC multidrug efflux pumps. Such understanding may prompt the use of completely new approaches to manipulating the function of these transporters in a way that is helpful for drug therapy. The use of modulators to block MDR during cancer chemotherapy treatment has been an attractive goal, however, the clinical trials carried out to date have proved disappointing. It is still not clear whether this approach has enough promise to be pursued in the future with the highly specific and efficacious third-generation modulators that have been developed recently. Naturally-occurring polymorphisms of the ABC multidrug efflux pumps have only been identified relatively recently, and much more work remains to be done to resolve some of the current controversies and determine their true impact on transporter function. The majority of studies in this area have suffered from serious experimental limitations, such as sample selection, sample size, confounding factors and genotype/phenotype errors, and a comprehensive set of recommendations to avoid these problems in the future has been presented [129]. The role of polymorphisms in responses to drug therapy and disease susceptibility is a developing field that will clearly be important in the future as the ultimate goal of personalized medicine is pursued.

It is possible that pharmacogenomics may, in the future, be able to predict individual responses to drug treatment, leading to improved treatment and clinical outcome.

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Executive summary

- Three ABC superfamily multidrug efflux pumps play a central role in protecting sensitive tissues from exogenous and endogenous toxic compounds: P-glycoprotein (Pgp, ABCB1), MRP1 (ABCC1) and ABCG2 (BCRP). These transporters show overlapping substrate specificity, and affect the uptake and distribution of many clinically important drugs.
- Pgp and the ABCG2 homodimer comprise two transmembrane (TM) domains and two nucleotide binding domains (NBDs), whereas MRP1 has an extra N-terminal TM domain. The NBDs likely dimerize during the catalytic cycle to form a nucleotide sandwich with two bound ATP molecules, whose hydrolysis drives active drug transport.
- Substrates for Pgp and ABCG2 are lipid-soluble, and they are likely expelled from the membrane into either the extracellular aqueous phase (hydrophobic vacuum cleaner) or the outer membrane leaflet (drug flippase).
- Many chemical modulators that inhibit the transport activity of Pgp are known, while fewer have been identified for MRP1 and ABCG2; however, only a small number of these compounds are suitable for clinical use.
- Expression of ABC drug transporters in human tumors has been linked to resistance to anticancer agents and poor prognosis, however, attempts to inhibit their activity in clinical trials using modulators have been disappointing.
- Polymorphisms in ABC efflux pumps have been implicated in different responses to chemotherapy and drug therapy, as well as disease susceptibility. However, overall results have been inconclusive, and the impact of genotype on the expression and function of these transporters remains controversial.

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