Part One:
Human Transporter Families – Structure, Function, Physiology

Transporters as Drug Carriers: Structure, Function, Substrates. Edited by Gerhard Ecker and Peter Chiba

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The ABC Transporters: Structural Insights into Drug Transport*

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Abbreviations

ABC	ATP binding cassette
ATP	adenosine triphosphate
CFTR	cystic fibrosis transmembrane conductance regulator
CsA	cyclosporin A
ICL	intracytoplasmic loop
MRP	multidrug resistance protein
NBD	nucleotide binding domain
NCS	noncrystallographic symmetry
P-gp	P-glycoprotein
SUR	sulfonylurea receptor
TMD	transmembrane domain

1.1
ABC Proteins – Structure and Function

1.1.1
ABC Proteins

A superfamily of membrane proteins involved in transport has been designated the ATP binding cassette (ABC) family [1]. About 5% of the entire *Escherichia coli* genome encodes components of ABC transporters [2]. Their existence across all the domains of life is an indication of their wide physiological roles, and in humans they are associated with several diseases [3]. The members of this superfamily bind ATP and typically use the energy from hydrolysis to translocate a wide range of substances (including sugars, amino acids, glycans, sterols, phospholipids, peptides, proteins, toxins, antibiotics, and xenobiotics) across cytoplasmic and organellar mem-

*This chapter is dedicated to the memory of Alhaji Bukar Kamis.

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branes [4–8]. In addition to the export and import functions, ABC transporters are known to function as receptors and channels and to mediate other physiological phenomena [4, 9]. The transporters appear to be always unidirectional (see Ref. [10]); but in bacteria, they are either importers or exporters while in eukaryotes they are exclusively exporters (so far) [7].

1.1.2 Predicted Topology of ABC Proteins

Topology prediction indicates the existence of transmembrane domains (TMDs) with between 4 and 10 (TM) α-helices and cytosolic ATP binding/hydrolysis domains designated nucleotide binding domains (NBDs). The minimal structural requirement for an active prokaryotic and eukaryotic transporter is thought to consist of two TMDs and NBDs each (Figure 1.1) [11–13]. This minimum requirement can be satisfied by a single polypeptide chain (full transporter) or can be assembled from two equal (homodimeric) or unequal (heterodimeric) polypeptide chains (half transporters). The assembly of one full transporter protein can be from four, three, two, or one separate polypeptide. Some ABC transporters are observed to possess an additional TMD and also soluble regulatory domains associated with the NBDs. Many bacterial importers have an accessory substrate binding protein (SBP).

1.1.3 Nucleotide Binding Domains

ABC transporters use the energy of nucleotide hydrolysis to transport substances across membranes against a concentration gradient [14]. All ABC proteins have

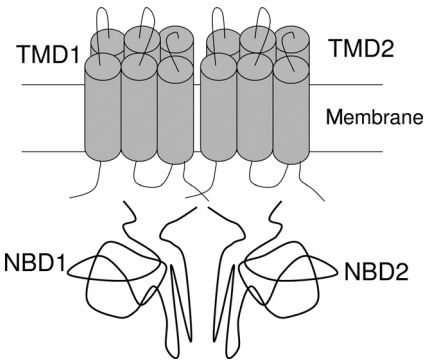

Figure 1.1 Schematic depiction of the organization of ABC proteins. The minimal structural unit appears to consist of two integral transmembrane domains, each with (typically) six helical transmembrane spans, and two extrinsic nucleotide binding domains. At the amino acid sequence level, the nucleotide binding domains are relatively well conserved across the domains of life compared to the transmembrane domains.

two cytoplasmic nucleotide binding domains containing conserved sequence motifs for binding/hydrolysis of ATP; this can be thought of as a standard engine that is bolted onto a specialized translocation pathway – formed by the TMDs [15]. In some prokaryotic NBDs, both are functionally identical and presumably contribute equally to the protein activity, whereas in eukaryotes, the NBDs are often functionally nonequivalent. Deletion of either NBD abolishes transport [14, 16–24], although recently Riordan and colleagues showed that NBD2 was partly dispensable for channel activity in mammalian CFTR [25]. Senior and colleagues proposed that ATP hydrolysis at the two NBDs could take place in an alternate fashion [26], and other workers have argued for functional symmetry [27–29] or asymmetry [30–34].

1.1.3.1 Conserved Motifs of NBDs

ABC transporters translocate many different allocrites (transport substrates), but the primary structures of the NBDs show ~25% sequence identity across the whole superfamily [9]. Marked sequence conservation is observed over five short regions found in the NBDs: (i) the Walker A and (ii) Walker B regions, which are separated by approximately 90–120 amino acids and between which lie the (iii) "signature" motif [2, 7, 9, 35, 36] and (iv) the glutamine loop (Q-loop) [37]. The most C-terminal motif is the histidine loop (H-loop) [2]. The signature, Q-loop, and H-loop seem to be specific to ABC transporters [9], and the function of these is described in more detail below. A recent description of the importance of a highly conserved aromatic residue has led to the naming of an "A-loop" at the N-terminus of the NBD [38].

1.1.4 Transmembrane Domains

The primary function of the TMDs is to provide a pathway through which allocrites can cross the membrane. It is not difficult to imagine how such a pathway could readily be adopted for channel function (e.g., CFTR), but for receptor-type ABC proteins (such as SUR1), the evolutionary step is less obvious. However, for all these functions, the TMDs appear to be the main determinants of specificity [3, 39, 40]. TMDs are much more variable in their amino acid sequences than the NBDs [15, 41]. Hydropathy predictions, which have so far been borne out by later structural studies, imply multiple transmembrane α-helices, typically six per domain (see Section 1.2), but with many exceptions [3]. Eukaryotic ABC proteins in the C class (ABCC subdivision) frequently contain a whole extra TMD with five predicted transmembrane spans [7].

1.1.5 Mechanisms of Transport

It is a general assumption that the TMDs provide the pathway for translocation of allocrites. The crystal structure of a prokaryotic ABC transporter (BtuCD) revealed that the TMDs create an outward-facing cavity at the interface between the two (BtuC)

subunits. It was postulated that alternate opening and closing of this cavity to the periplasmic and cytoplasmic sides could allow translocation [15, 42]. The recent structure of a related *Haemophilus influenzae* ABC protein, which shows a similar central cavity but is more open on the inner facing (cytoplasmic) side, has added strength to this hypothesis. However, in neither case was allocrite bound to the protein [43]. On the other hand, a structure with allocrite bound was first published for the bacterial lipid A exporter/flippase MsbA [44]. In this structure, the (two, NCS-related) lipid A molecules were situated on the outside of the protein on the extracellular side, rather than at the interface between the two TMDs. Whether this configuration represents an end point of the translocation pathway or perhaps indicates translocation pathways on the outside rather than the inside of this transporter remains to be established. It was possible that the location of the lipid A molecules may be determined by crystal packing constraints rather than representing the physiological binding sites. However, this and earlier structures of MsbA were removed from the protein data bank (www.rcsb.org), and the papers describing them were retracted [45, 46]. Revised structures for MsbA were later published [47], but in the new manuscript and deposited atomic coordinates, the bound lipid A molecules were not present. Recently, however, the first information on the mechanism and location of allocrite binding has emerged with the publication of the structure of the maltose transporter $MalFGK_2$ [48] with its cognate periplasmic substrate binding protein. These data show that maltose is bound in an outward-facing crevice formed between the two transmembrane proteins (MalF and MalG). This crevice is capped at the top by the SBP, the maltose binding protein, thus forming a cavity in which the allocrite is sequestered. Low- and medium-resolution structures determined for P-gycoprotein (P-gp) and MRP1 also suggested the existence of a central cavity [3, 49–51]. However, findings indicating the existence of two or three allocrite binding sites for P-gp suggest that a single translocation pathway model may be too simplistic [40, 52–56]. That P-gp has a common site for structurally unrelated allocrites may best be explained by allocrite-induced fit [56–58], implying that the translocation pathway may be relatively adaptable. P-gp has also been reported to possess at least one regulatory site [59, 60] in the TMDs.

1.1.6
Energy for Translocation

Early structural studies of NBDs in isolation revealed at least three different possibilities for dimer organization [9, 37, 61–63]. However, accumulating evidence now strongly suggests that the NBDs form a sandwich dimer in which the two ATP molecules are concertedly bound at the NBD interface formed between the Walker A, B, and signature regions [15, 64]. The question remains, especially in eukaryotes, as to the functional equivalence or asymmetry of the NBDs. Several findings suggest that the two NBDs may be functionally equivalent, with one NBD undergoing catalysis at any given point in time (see Section 1.3). However, biochemical and mutational studies have indicated that NBDs of P-gp, MRP1, and CFTR are functionally distinguishable [18, 19, 32, 65–69].

1.1.7
Coupling of ATP Hydrolysis to Transport

The coupling of nucleotide binding/hydrolysis in the NBDs to allocrite transport in the TMDs is envisaged as a coupling of conformational changes in each half of the ABC proteins. Mutational analysis of homodimeric LmrA showed that disruption of NBD inhibited not only the ATPase activity but also the transport function of the protein suggesting interactions between NBDs and TMDs [70–73]. These findings are in agreement with studies of P-gp where ATP binding/hydrolysis and vanadate trapping have profound effects on allocrite binding affinities [40, 74–80]. The switch from high to low affinity for allocrite is probably an integral part of transport/release, and coupled conformational changes could easily bring about such changes in affinity. High-resolution studies of NBD–NBD interactions in the presence or absence of nucleotide suggest small but significant adjustments in the crucial NBD helical regions that interact with the intracellular loops of the TMDs [81]. Low-resolution electron microscopic studies have implicated large conformational changes in the TMDs upon nucleotide binding [50] suggestive of a significant gearing (or amplification) of the nucleotide-induced conformational shifts as they propagate into the TMDs.

It has been observed that ATP binding decreases allocrite binding affinity [76, 77, 82] indicating the precedence of allocrite binding over ATP binding. Similarly, allocrite binding was found to enhance NBD–NBD cross-linking [57, 83, 84] suggesting crosstalk between allocrite binding at the TMDs and the proximity of the NBDs. A model where allocrite binding promotes dimerization of the two NBDs and increased ATP binding is attractive, since it allows allocrite regulation of potentially wasteful ATPase activity [85]. Structurally, one might expect the signal transmission interface between NBDs and TMDs to be highly conserved [15, 57, 83, 84], but so far there is no obvious switch region emerging from the few structures available: For BtuCD and the related HI1470/1, a single loop extends down from the TMDs into the NBDs. This "L-loop" (sometimes also termed the "EAA" loop) in BtuC is close to the BtuD Q-loop, a proposed sensor for γ-phosphate that might change its conformation upon nucleotide binding/hydrolysis [15, 86, 87]. There is some evidence that mutation around the signal transmission interface could perturb assembly as well as the coupling of ATP binding/hydrolysis to allocrite transport [15, 21, 88, 89]. However, in the Sav1866 structure, and as far as we can tell in the MsbA structures, the TMD/NBD interface is much more extensive and involves at least two loops of the TMDs. In the Sav1866 structure, one of the loops is domain swapped with the opposing NBD, inextricably linking the four components of the structure [90].

1.2
Structures of ABC Transporters

1.2.1
Tertiary Structure

Despite technical difficulties, a number of ABC transporters have now been over-expressed and purified sufficiently for 3D crystallization trials [42, 82, 91–94]. The

emergence of a wider range of crystallization techniques for membrane proteins has also been a positive development [95, 96]. In addition, electron microscopy approaches have yielded low- to medium-resolution structures.

The first high-resolution structure of the NBD domain of HisP (histidine permease) from *Salmonella typhimurium* was reported in 1998 [62]. In addition, high-resolution structures of RbsA from *E. coli* [97], MalK from *Thermococcus litoralis* [37], MJ0796 from *Methanococcus jannaschii* [87], MJ1267 *Methanococcus jannaschii* [98], Rad50 from *Pyrococcus furiosus* [63], SMC from *Thermotoga maritima* [99], MutS from *E. coli* [100, 101], human Tap1 [102], GlcV from *Sulfolobus solfataricus* [103], HlyB from *E. coli* [104], and human CFTR [105] were determined shortly thereafter. A recent survey of the protein data bank yielded >160 structures, nearly all for NBDs. For such a large data set, structural bioinfomatic studies are now required, but in essence the tertiary structures of the NBDs all have a two-domain organization consisting of catalytic and signaling domains [61]. The catalytic domain houses the Walker A and B motifs and a β-sheet region that interacts with and positions the base and ribose sugar of the nucleotide as well as phosphate moieties [106]. The signaling subdomain contains mostly α-helices that houses the C-loop or ABC signature sequence, and it is this subdomain that has been thought to interact with the TMDs [61, 106].

However, as discussed above, functional insights from these structural studies crucially depend on elucidating the quaternary structure of these proteins [107, 108], and data on this level of structural organization are rare and significantly harder to obtain. Nevertheless, the sheer abundance of ABC proteins has made them particularly attractive for structural proteomics projects, and in many countries (the United Kingdom being a notable exception), funding for structural proteomics of ABC proteins has been forthcoming, leading to significant advances.

The first data for an entire ABC protein emerged from a structural proteomics screen of ~50 *E. coli* ABC proteins [94] (revised in Ward *et al.* [47]). The data for a lipid transporter/flippase termed MsbA revealed a homodimeric cone-shaped protein with base dimensions of 120 Å × 115 Å × 64 Å, with an unusual tilting of the six TMD α-helices to about 30–40° from the normal to the membrane and making monomer–monomer contacts only at the extracellular side [94] (revised in Ward *et al.* [47]). The resulting effect of this tilt is the wide separation of the NBDs by about 50 Å. Being the first crystal structure of an ABC transporter protein, several conclusions were drawn [82, 106]. The structure not only confirmed several previous biochemical findings, including the prediction of six transmembrane α-helices, but also raised numerous questions [82, 106]. Most significant was the observation that the two NBDs were separated by ~50 Å, which contradicted previously observed NBD interactions (fluorescence resonance energy transfer measurements and other cross-linking studies) [9, 109]. Subsequent searches for homologues led to crystal structures of MsbA from *Vibrio cholerae* and *S. typhimurium* (see Figure 1.2), which provided some explanations [44, 93, 94] (all revised in Ward *et al.* [47]). Structural similarities within each MsbA monomer were immediately apparent, but the quaternary structure differed considerably, especially in the orientation of NBDs [93, 94] (both revised in Ward *et al.* [47]). The revised MsbA structures have been corrected for handedness and tracing of the polypeptide chain in the TMDs, but the *E. coli* MsbA structure still has widely separated NBDs.

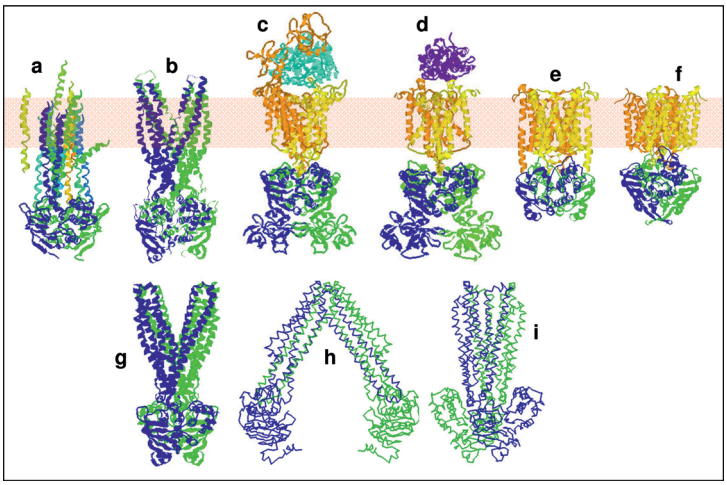

Figure 1.2 Comparison of high-resolution (b–i) and medium-resolution (a) structures of ABC proteins. The predicted transmembrane regions are indicated by the pink boundary for (a)–(f). (a) Medium-resolution model of P-gp with α-helices placed in elongated elements identified in the EM map [110]. NBDs are based on the MJ0796 structure [87] that was manually docked into a bilobed density in the appropriate region of the medium-resolution 3D map. (b) Sav1866 homodimer [90]. (c) $MalFGK_2$ with the periplasmic maltose binding protein (cyan) [48]. (d) ModABC, with the periplasmic binding protein (purple) [111]. (e) BtuCD [42]. (f) Putative metal chelate transporter H10796 [43]. (g–i) MsbA from *S. typhimurium*, *E. coli*, and *V. cholerae*, respectively [47]. Structural similarities are identifiable in the trans-membrane regions of (b) with (g), (c) with (d), and finally (e) with (f).

Another structural proteomics screening of 28 bacterial ABC transporter proteins yielded a second structure (Figure 1.2): the vitamin B12 importer (BtuCD) from *E. coli* [42]. The transporter consists of two subunits BtuC (TMD) and BtuD (NBD) with overall dimensions of 90 Å × 60 Å × 30 Å. Extensive contacts between the subunits [42], and the juxtapositioning within the NBDs of the ABC signature sequence and P-loop (involved in signal transduction and nucleotide binding, respectively), suggested that the BtuCD structure was more consistent with the available biochemical information for ABC proteins. Paradoxically, though, the BtuCD protein with 10 transmembrane α-helices per TMD appears to be very different from eukaryotic ABC proteins and hence has proven less amenable to homology modeling than the MsbA structures and the recently published Sav1866 structure (Figure 1.2) [90]. The interface between the BtuC subunits is formed by the antiparallel packing of two pairs of helices creating a cavity that opens to the periplasmic space and closes on the cytoplasmic side by residues Thr142 and Ser143 in the loops connecting helices 4 and 5 [15, 42, 82]. In BtuC, there is a prominent cytoplasmic loop between helices 6 and 7 folding into two short helices (L1 and L2) making extensive contacts with BtuD (NBD) [15, 42]. Some sequence alignments have suggested architectural conservation especially in helix L2 [15, 82]. For example, this region has been proposed to correspond to the first cytoplasmic loop of drug exporters and to intracellular domain 1 (ICD1) of EC-MsbA [15, 82]. This loop is also often termed the "EAA" loop (see below).

A surprising facet of ABC proteins is the structural plasticity of the transmembrane regions. From the six ABC proteins for which high-resolution structures are available, there are three almost entirely different "folds" for the transmembrane domains (i.e., they are threaded through the membrane on different paths). This is in stark contrast to the NBDs, where structural homology is very clear, and the similarities extend even to the NBD–NBD association in the intact transporter (with the exception of the lower resolution *E. coli* and *V. cholerae* MsbA structures) (see Figure 1.2). In one group of exporters, exemplified by Sav1866 and *S. typhimurium* MsbA (Figure 1.2b and g), two lots of six transmembrane α-helices form the homodimeric ABC protein. Four of the six transmembrane α-helices (TM2–5) are very long (~70 Å) and have significant helical extensions on the cytoplasmic side, which constitute the so-called intracytoplasmic loops (ICLs). These loops contact the NBDs, presumably act as an interface communicating structural changes in the NBDs to the TMDs, and separate the NBDs from the membrane surface. Intriguingly, the second ICL, joining TM4 and TM5 contacts the opposite NBD to the one contacted by the first ICL. This "crossover" (sometimes termed a domain swap) is mirrored on the extracellular side of the TMDs where the extracellular sides of TM1 and TM2 make contacts with the opposite polypeptide subunit [47, 90].

The second "fold" has been observed with the importers BtuCD and HI1470/1 [42, 43] where a large number (10) of short transmembrane α-helices exist for each TMD. The ICLs for this fold are also very short, with only one loop making significant associations with the underlying NBD. This loop has been termed the "EAA" loop on the basis of this relatively three-residue motif conserved within it. Although there is some "crossover" of contacts in the TMDs, there is no evidence for this in the ICLs. This TMD fold seems (so far) the most distant from the eukaryotic ABC proteins, and hence perhaps the least useful for homology modeling.

The third fold is exemplified by the importers ModBC, MetNI and $MalFGK_2$ [48, 111, 248]. Similar to the second type of fold, the helices are short and there is hardly any ICL region apart from a single loop (similar to the EAA loop described above) that contacts with the underlying NBD. At first sight, the MalFG subunits, with eight and six transmembrane spans, appear quite different from the ModB subunit with six transmembrane spans. (*Note:* The term "helix" is not used for this group as some of the transmembrane spans are composed only partly of α-helices, and also regions of the subunits apparently dip into the membrane without spanning it.) However, when ModB is aligned with MalF and MalG using the "EAA" loop as an anchoring point, the six ModB transmembrane spans superimpose with reasonable precision on the equivalent regions in the MalF and MalG subunits. Hence, a core in the fold for the TMDs of ModBC and $MalFGK_2$ can be identified. Davidson and colleagues have also noted that the (substrate-lacking) ModBC structure appears to be more open on the cytoplasmic side of the membrane, the reverse being the case for the substrate-containing $MalFGK_2$ structure (Figure 1.3). Whether this reflects conformational changes in the TMDs associated with substrate transport still remains to be established.

Transmission electron microscopy (TEM) studies of ABC proteins have also provided insight into the quaternary structure and also served as a useful tool

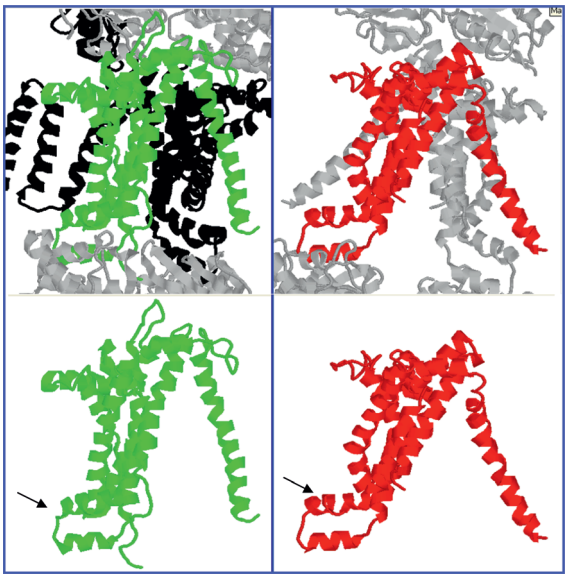

Figure 1.3 Comparison of the transmembrane regions of the MalFGK$_2$ transporter (upper left panel) and the ModBC transporter (upper right). For the former, the MalG subunit is in green, the MalF subunit in black. For the latter, one ModB subunit is highlighted in red. For both transporters, the NBDs (cytoplasmic side) are toward the bottom, while the periplasmic substrate binding subunits are uppermost. The ModBC structure (right) has a more "open" structure on the cytoplasmic side. The lower panels show a simplified representation of the MalG (left) and ModB (right) subunits in the same orientation as in the upper panels, illustrating the similarity in the folds of the two proteins when aligned on the basis of the EAA loop (arrow).

for identifying conformational changes accompanying nucleotide binding (see Figure 1.4).

However, only one of these TEM studies (for P-gp) has so far yielded data at a resolution sufficient for the identification of transmembrane α-helices and other subdomains [51]. Nevertheless, these data were sufficient to confirm the conservation of overall tertiary structure between prokaryotic and eukaryotic ABC proteins that was predicted from the analysis of their primary structure (see Figures 1.2 and 1.5). Indeed, a close examination of the structures suggests that while all display similar NBD folds (Figure 1.2), the transmembrane region of the single eukaryotic example (P-gp) has a closer similarity to Sav1866 and *S. typhimurium* MsbA (revised) than to BtuCD, especially in the spacing of the NBDs from the TMDs by the cytoplasmic helical loop regions.

1.2.2
Quaternary Structure of ABC Proteins

The oligomeric state of detergent-solubilized and purified membrane proteins can depend on a variety of factors such as the detergent used in the purification

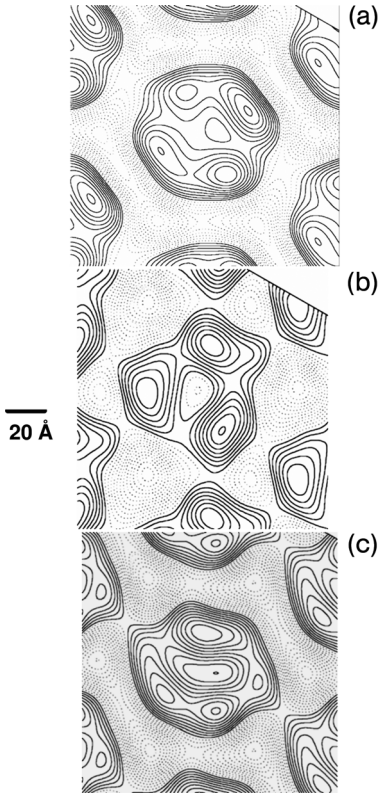

Figure 1.4 Low-resolution projection maps of P-gp obtained from 2D crystals in the nucleotide-free state (a) and in the presence of nucleotides (AMP-PNP) (b) and ADP/vanadate (c). These 2D maps suggest, but do not unequivocally demonstrate, major conformational changes associated with the binding of nucleotides. Major molecular rearrangement between the AMP-PNP-bound state and the nucleotide-free state was later confirmed by generating 3D structures for them. The binding of ADP/vanadate produced only slight changes compared to the nucleotide-free form (a and c), and so far no 3D structure has been generated for that condition. Hence, for the ADP/vanadate trapped P-gp, it is possible that the changes in the projection map are due to differences in crystal packing rather than whole-scale conformational rearrangements in the protein.

and the degree of overexpression of the recombinant protein that may facilitate quaternary interactions [112]. There have been various oligomeric states assigned to different eukaryotic ABC transporters, and no clear consensus is emerging; indeed, entirely different oligomeric forms could exist for the same transporter protein. Bacterial examples of crystallized ABC proteins exist in a form that would be consistent with a single functional transporter – equivalent to a monomer for most eukaryotic ABC proteins where a single polypeptide encodes both TMDs and both NBDs [42, 43, 47, 48, 90, 111]. Similarly, P-gp and CFTR form epitaxial crystals grown in elevated salt and polyethylene glycol, which are composed of monomeric proteins. The only possible exception to this uniformity is the crystalline *E. coli*

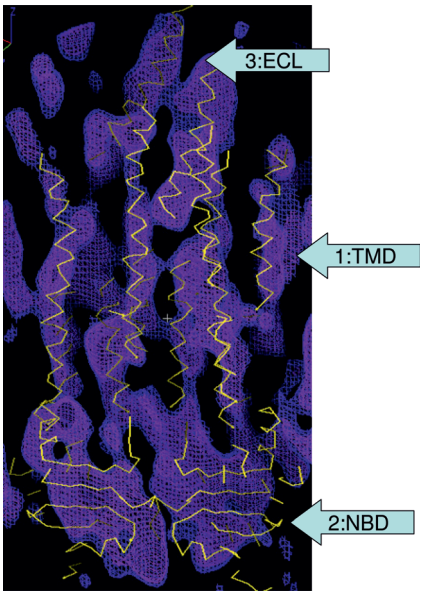

Figure 1.5 Central section through the medium-resolution 3D density map of P-gp in the presence of nucleotide as determined by electron microscopy of 2D crystals. 1: The long cylindrical densities that extend down into the putative NBD region were fitted as simple α-helices. 2: The two heart-shaped lobes of density at the bottom of the map have been fitted using the MJ0796 NBD dimer (with nucleotide bound). Note that individual β-strands cannot be resolved at this resolution (8 Å). 3: Small regions of density extending at the top of the map, presumably extracellular loops and/or glycosylation, have been fitted using short α-helices.

MsbA structure where contacts between the two polypeptides are sufficiently peripheral to suspect that this structure represents a state equivalent to half a functional transporter [47]. However, noncrystalline specimens of eukaryotic and prokaryotic ABC proteins appear to contain both monomeric and dimeric particles [3, 51, 110, 113–117]. Ni-NTA nanogold labeling of particles of these two proteins was also carried out, allowing their C-terminal polyhistidine tags to be localized within the low-resolution structures [113, 117]. This labeling was not able to directly confirm the dimeric nature of the particles, however, since the gold labeling efficiency (<25% of particles were labeled) ensures that few particles are doubly labeled (see Figure 1.6).

Pdr5p, a multidrug exporter in yeast, when examined by EM and single particle analysis, also revealed dimeric (i.e., dimer of homodimer) particles similar to the arrangement observed for P-gp and CFTR [116]. Freeze-fracture electron microscopy of cells expressing high levels of P-gp displayed the presence of large particles and density gradient centrifugation indicated oligomers for the detergent-solubilized protein, although to some extent these, and similar studies with CFTR, may be criticized because of the assumptions made regarding the identity of the particles observed by freeze-fracture and atomic force microscopies. Some oligomeric associations that have been observed are unambiguously the product of the isolation

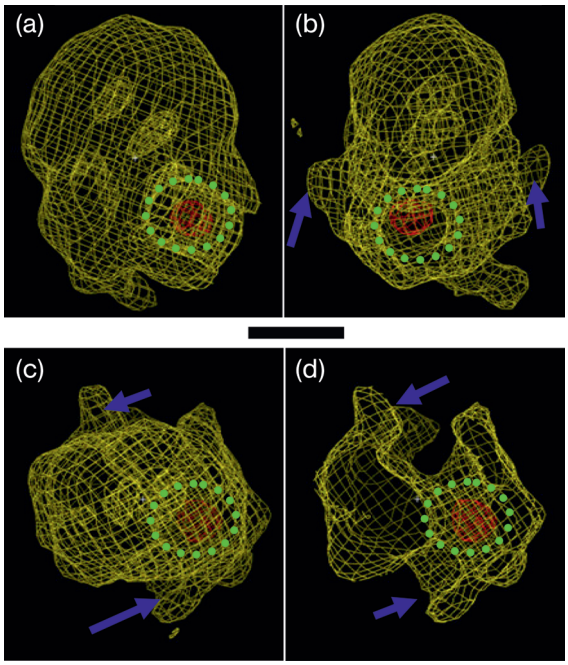

Figure 1.6 Low-resolution structure of human, recombinant, 6-His-tagged P-gp particles labeled with 1.8 nm diameter Ni-NTA nanogold. The structure (yellow netting) shows overall dimensions of 120 Å × 100 Å × 70 Å, sufficient to enclose two P-gp molecules. Panels (a) and (b) show side views orthogonal to each other, while panel (c) shows a view from the top after rotation by 90° around the horizontal axis. Panel (d) is the same view as in panel (c), but with the top half of the structure stripped away. Indicative of a dimeric organization, symmetry-related "arms" (blue arrows) protrude on either side of the structure. The Ni-NTA nanogold (red netting, green circle) breaks the twofold symmetry, probably because the labeling efficiency is only ~25%; hence, few particles are double labeled. The gold sphere is bound on the lower half of the structure, allowing its designation as the NBD region. Scale bar = 40 Å.

procedure, such as the bacterial YvcC protein (now termed BmrA), which after partial reconstitution with lipid forms a 24-membered ring with YvcC proteins forming spokes emanating from the center of the ring [115]. On the other hand, the complex formed between the Kir6.2 potassium channel and the SUR1 ABC protein, which has been observed as an octameric complex (tetramer of heterodimers), seems reasonable given the function of the complex, with four SUR1 proteins arranged around the tetrameric potassium channel [118]. A tetramer of similar external dimensions, but lacking the central potassium channel, has also been observed in EM studies of the isolated multidrug transporter ABCG2^{R482G}, otherwise known as the breast cancer resistance protein [112]. The tetrameric association in both cases appears to be mediated strongly by contacts between adjacent NBDs in separate monomers in the complexes.

The only exception to the general observation of multiple association of purified ABC proteins (so far) is the yeast ABC protein Mdl1 [119], where studies of

noncrystalline specimens by EM were interpreted as showing single transporters containing two polypeptides (each containing one TMD and one NBD). However, the overall dimensions of these particles (~10 nm × 10 nm × 12 nm) suggest that two transporters (four polypeptides) could have been fitted to the 3D density map. A major caveat with all these structural studies is that nonphysiological oligomeric associations could form upon solubilization of the proteins from the membrane and/or during purification in detergent micelles. The availability of structural data for ABC proteins reconstituted back into a lipid bilayer system is very limited at present [50, 115], and clearly there is a need for further structural studies of such reconstituted systems if we are to understand better the true nature of conformational changes and oligomeric associations in ABC proteins.

1.3 Multidrug Resistance and ABC Transporters

Cellular resistance to antibiotic and anticancer drugs is considered to be a major factor for the treatment of infectious diseases or cancers [120, 121]. Sometimes, microorganisms and cancer cells exhibit cross-resistance to a wide variety of functionally and structurally unrelated drugs [121, 122]. This phenomenon is termed multidrug resistance (MDR), a concept introduced into scientific literature in the 1970s [123]. One of the most important mechanisms of multidrug resistance is via active drug export from the cell mediated by membrane proteins [121, 124, 125].

The multidrug transporters are divided into two broad classes, primary and secondary active transporters, although they both mediate the translocation of a range of substances across membranes [121]. Secondary active transporters are predominant in prokaryotic organisms and mediate the drug efflux reaction in a coupled exchange with sodium ions or protons [125, 126]. Primary active transporters use the energy of ATP hydrolysis to translocate compounds and play a key role in eukaryotic drug resistance and to varying degrees in bacteria, fungi, and protozoans [121]. One of the best characterized bacterial proteins, LmrA, shows about 34% amino acid sequence identity with P-glycoprotein, which is probably the best characterized eukaryotic ABC protein [70, 73, 127]. Since the completion of the human genome sequence [128, 129], about 48 human ABC proteins have been identified and as many as 12 might be implicated in drug transport [7, 121].

1.3.1 P-Glycoprotein

1.3.1.1 Historical Background

Biedler and coworkers [123] reported that Chinese hamster cells selected for resistance to actinomycin D also showed cross-resistance to mithramycin, vinblastine, vincristine, puromycin, dauromycin, democolcine, and mitomycin C. Thus, upon selection with a single cytotoxic drug, mammalian cells became simultaneously cross-resistant to a range of drugs with different chemical structures and molecular

mechanisms of actions [123, 130–132], a phenomenon referred to as multidrug resistance [123, 132]. Initially, it was believed that the resistance was due to a membrane alteration that reduced the rate of permeation [133]. However, Juliano and colleagues [133] revealed that drug-resistant Chinese hamster ovary cell membranes contain a cell surface glycoprotein of about 170 kDa that was not observed in drug-sensitive cells. This remarkable observation, achieved well before proteomic analysis became possible, was possible because of the unusually high expression of this protein, which was named P-glycoprotein (where P stands for permeability).

There is a significant body of literature on the biochemistry and pharmacology of P-gp. The recognition that gene amplification and overexpression of the protein could give rise to the multidrug resistance phenotype [134, 135] was followed by the cloning and sequencing of the cDNA that encoded the protein [136–140]. The transfection of the cDNA into cells followed by selection for drug resistance provided an early means of isolating P-gp for functional studies [141]. The deduced amino acid sequence predicted 12 transmembrane α-helices, 2 nucleotide binding domains, and 3 potential glycosylation sites within the 1280 amino acid residues [88, 136]. Biochemical, biophysical, genetic, and microscopic analyses have all been used to investigate the mechanistic behavior of the protein. Cross-linking experiments and pharmacokinetic studies of the proximities of some transmembrane helices have led to speculation on the location of drug binding sites and the number of sites. Some of these studies are described in more detail below.

1.3.1.2 **The Role of P-gp in Drug Resistance**

One of the major difficulties in cancer chemotherapy is the development of multidrug resistance, a phenomenon of cross-resistance to an array of drugs. Since the discovery of P-gp on the cell surfaces of tumor cells with a wide range of drug-resistant phenotypes [133] and subsequent findings from numerous other laboratories, it has been considered that P-gp when overexpressed in tumor cells can mediate the ATP-dependent extrusion of a variety of drugs, concomitantly reducing intracellular accumulation [141–148].

It is indeed observable that P-gp expression contributes to multidrug resistance. However, establishing a direct and simple relationship between P-gp expression and multidrug resistance is difficult due to differences in populations of tumor cells and methods of measuring P-gp expression [149]. The observation that most tumors contain heterogeneous cell populations with varying degrees of P-gp expression may lead to over/underquantification in various cell populations [150]. Finally, it has been emphasized that only some tumors express P-gp [131].

Although it is apparent that P-gp lowers the concentration of many anticancer agents in tumor cells, the extent of the reduction due to P-gp function alone is often unclear. A recent study investigating the role of P-gp in paclitaxel concentration in tumor cells by comparing the relative importance of extracellular drug concentration, P-gp efflux rate, binding affinity to tubulins/microtubules, and intracellular contents of tubulin proteins indicated that the role of P-gp in multidrug resistance might be less significant compared to other biological factors [131, 151]. The rank order of importance of these factors was reported to be extracellular drug concentration

intracellular binding capacity > intracellular binding affinity > P-gp-mediated efflux [131, 151]. It is the conclusion of the above-mentioned authors that the delivery of paclitaxel to tumor cells rather than other mentioned factors determines intracellular drug concentration. This report was supported by a study that showed intravenous administration of radiolabeled daunorubicin to rats bearing bilateral tumors indicated that P-gp accounted only for partial drug resistance [152]. Nevertheless, poor brain penetration of radiolabeled drugs demonstrated a very significant role for P-gp [152, 153]. Despite all these arguments, the general conclusion still remains that P-gp is a major player in multidrug resistance. Therefore, the development of relatively potent but nontoxic P-gp inhibitors could greatly reduce its effect on drug accumulation in cells.

In addition to the significant role P-gp plays in multidrug resistance, multidrug resistance protein 1 (MRP1) is another important ABC transporter protein with similar properties. MRP1 was identified in 1992 as a second drug transporter in humans [154]. It can confer resistance to a variety of drugs when overexpressed in cells. MRP1 has been implicated in the transport of etoposide, teniposide, doxorubicin, vincristine, leukotrienes, glutathione conjugates, glucuronides, and sulfates [155–157]. Like P-gp, MRP1 is thought to provide protection to normal tissues [158, 159]. For other members of the MRP family, such as MRP2, MRP3, MRP4, MRP5, MRP6, ABCC11, and ABCC12, links to multidrug resistance are less well defined [158].

1.3.1.3 Tissue Distribution and Physiological Roles

MDR genes are expressed in normal tissues, prompting researchers to elucidate their physiological roles. Mice lacking P-gp genes (mdr1a and mdr1b) have a subtle phenotype [160] indicating a role for P-gp in physiological defense against xenotoxins. The polarized pattern of P-gp expression in many cells supports this probable role. Thiebaut *et al.* [161] reported the localization of P-gp in the epithelial cells of excretory organs such as the bile canalicular membrane of hepatocytes in the liver, proximal tubules in the kidney, and enterocytes lining the wall of the intestines. The presence of P-gp in the capillary endothelial cells in the brain and testes indicates other roles of significance in biology [162–164] (Sugawara *et al.*, 1990). The expression of P-gp in tissues that partake in steroid hormone biosynthesis is suggestive of its importance in production and secretion of cortisol and other steroids [161, 165–167]. P-gp also occurs in the placental trophoblasts from the first trimester of pregnancy to full term indicating a probable (protective) role in fetal development [167]. Hemopoietic progenitor cells are also shown to contain P-gp [168] where a role in protection against mutagens and teratogens seems likely.

1.3.2 Conformational Changes in the Mechanism of P-gp

It is apparent that if we are to circumvent the unwanted actions of P-gp in cancer chemotherapy, we need to understand the molecular basis of multidrug export. This requires us to understand both the structure and the mechanism of the

protein. It is widely accepted that the coupling of ATP binding and hydrolysis at the NBDs to the transport of allocrite in the TMDs could be mediated by conformational changes at different stages in the catalytic cycle. Recent findings seem to highlight the role played by dimerization and dissociation of the two NBDs in bringing about a change in conformation needed for translocation. For P-gp, several experimental approaches have been used to establish the existence of these changes, for instance, the use of differential tryptic digest patterns [169, 170], fluorescence quenching [171, 172], IR spectra [173], and monitoring of changes in accessibility of extracellular antibody (UIC2) epitope [50, 174–176]. The question remains as to what mediates these changes. Recent reports indicate that two molecules of ATP interact at the interface of the Walker A in one NBD and the LSGGQ signature motif in the other NBD and also that allocrites that stimulate or inhibit ATPase activity can cause the above-mentioned sequences to come closer or to move farther apart, respectively [83, 84]. These workers postulated that the LSGGQ sequence conveys the signal of conformational changes from the allocrite binding site to the ATP binding sites. This suggests that the architectural position of the LSGGQ sequence (influenced by allocrite) to Walker A is responsible for influencing the rate of ATPase activity that can be viewed as allocrite-induced conformational crosstalk between NBDs and allocrite binding site [57, 83, 84].

Even though a high-resolution crystal structure was not available for P-gp until recently (see Section 1.3.8), transmission electron microscopy of 2D crystals has yielded low- to medium-resolution 3D structures of P-gp. Conformational rearrangements were indicated in the low-resolution studies where two 3D structures were produced under different conditions – nucleotide-free and nucleotide-bound states [50, 51] (Figure 1.4). Studies of vanadate-trapped P-gp in the presence of ADP were also proposed to give a third conformational state, but no 3D structure has been generated so far for this condition [50]. Changes in the transmembrane region of the protein were particularly apparent. This work implicates major conformational rearrangements (i.e., observable even at ~20–25 Å resolution) in the transport cycle of P-gp. Higher resolution data for the nucleotide-bound form of P-gp [110] showed that asymmetry in the transmembrane region was mostly caused by different tilts of two of the helices (the remaining 10 helices showed roughly twofold symmetry). Since the nucleotide-free form of P-gp displayed a strong twofold symmetry [50], the authors speculated that the asymmetric tilting of the helices in the nucleotide-bound form could be a result of nucleotide binding at the NBDs. This putative conformational shift could perhaps be significant, since the asymmetry opens up one side of the transmembrane region that may allow access from and to the lipid bilayer. Interestingly, the (ADP-bound) Sav1866 structure also displays similar gaps open to the lipid bilayer on the side of the barrel of transmembrane helices, although in this case, symmetry is retained [90, 177]. Returning to an earlier question, this work also provides a plausible explanation for the evolution of receptor/switch functions of ABC proteins such as SUR1: Presumably, large-scale conformational changes in the TMD regions associated with nucleotide binding or release can be used to induce changes in a transducer protein (such as the Kir6.2 potassium channel).

1.3.3
Comparison of Sav1866 and P-gp Structures

The 3–4 Å resolution structure of the *S. aureus* putative multidrug transporter is the best homologue available for constructing models of eukaryotic ABC proteins such as P-gp [90, 177]. Similarly, the 8 Å resolution map of P-gp 2D crystals represented, until recently, the highest resolution data so far for any eukaryotic ABC protein, and thus a comparison between the two is appropriate. Qualitatively, this has been addressed in the preceding section's discussion of the gaps on the side of the barrel of transmembrane α-helices, but here an overall appraisal of correspondence is attempted. Sequence homology between Sav1866 and P-gp is low except in the conserved regions of the NBDs (see Section 1.3.1). However, the length and spacing of the hydrophobic transmembrane regions relative to the extracellular and intracellular loops are generally well conserved, suggesting that the overall fold of the two proteins may be qualitatively similar in the TMD regions. We therefore fitted the Sav1866 structure into the P-gp density map by hand using the *xfit* program within the *XtalView* software package, initially using the well-conserved NBD regions to guide the process, followed by fine-tuning the rotational and translational operations to give a good fit for the rest of the structure. The results of this exercise are summarized in Figure 1.7.

The views orthogonal to the long molecular axes in panels (a) and (b) confirm that the overall dimensions of Sav1866 fit well to the P-gp map, as expected from the sequence alignment. The distance from the bottom of the NBD (arrow 5) to the long extracellular loop between TM helices 1 and 2 (arrow 3) is ∼130 Å. This latter loop fits into a finger-like protrusion previously identified in the P-gp map as an extracellular domain, but a gap in the density (arrow 4) that may be due to local disorder precluded its assignment as a continuous loop. In contrast, the C2 symmetry-related loop on the other half of the Sav1866 homodimer is a poor fit to the P-gp map (arrows 1 and 2). It is tempting to use this observation to assign this section of the P-gp map to TMD2, which lacks the large extracellular loop of TMD1. As expected, the well-conserved NBD regions of Sav1866 dock into the lower portion of the map with a good overall fit and in a similar position to the MJ0796 dimer that was employed in a previous study [110]. The positions of bound ADP molecules in the Sav1866 structure are indicated by the dashed ellipses. The five-stranded parallel β-sheet, viewed edge-on, running between arrows 5 and 7, appears as a slightly curved slab of density in the P-gp map in panel (a) and has a characteristic heart shape viewed face-on (panel (b), arrow 8). A few discrepancies in this region are observed, however, such as the region of short helices and turns between residues 430–480 that contribute to the signature motif in Sav1866 (arrow 6a). Although this small region does not match density in one half of the map, its C2 symmetry-related equivalent fits well in the other half (see arrows 12a and 12b). Similarly, the region linking TM helix 6 to the start of the NBD in Sav1866 is poorly fit on one side of the structure (arrow 9a), while a good match is found on the other (arrow 9b). These various differences are probably due to the evolutionary divergence from twofold symmetry in P-gp versus the homodimeric Sav1866, although disorder in some regions is an alternative explanation. Presumably,

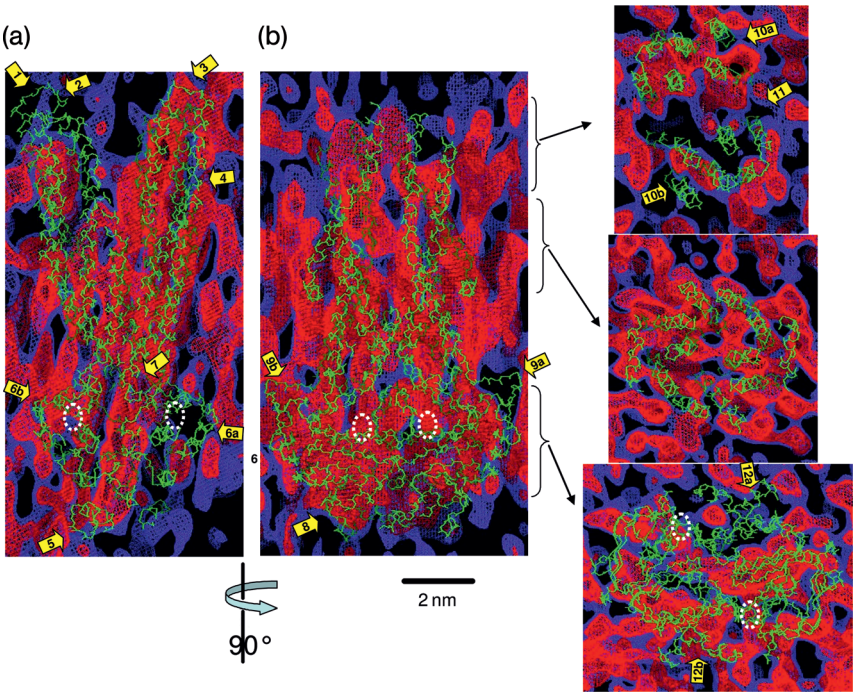

Figure 1.7 Comparison of the Sav1866 structure (green polypeptide Cα trace) and the 8 Å resolution map for P-gp (red and blue netting at 1σ and 1.5σ above the mean density level, respectively). Each panel represents a 25 Å thick slice through the center of the molecule (panels (a) and (b)), or slices perpendicular to the long axis of the molecule, as indicated by the brackets and arrows relative to panel (b). The positions of the two ADP molecules in the Sav1866 structure are indicated by the dashed ellipses. *Note:* One Sav1866 homodimer is shown docked into the center of a P-gp 2D crystal unit cell with a p1 plane group – additional densities around the edges of each panel arise from adjacent unit cells. See main text for description of the numbered arrows.

the presence of a linker region between NBD1 and TMD2 in P-gp also imposes some differences in the organization of the NBD region.

Slices through the map taken perpendicular to the long molecular axes further illustrate the similarities and differences between Sav1866 and the P-gp map. The extracellular transmembrane regions and loops that occupy two separate regions at the top of the Sav1866 structure correspond to two regions of density in the P-gp map (top right panel, arrow 10), which are separated by a low-density region. The correspondence on one side (arrow 10a) is better than that on the other (arrow 10b) where density is weak or lacking at the expected positions of two of the Sav1866 helices. There is a gap on one side of this region (arrow 11) that may allow access to the lipid bilayer in P-gp, as discussed previously [110]. A slice through the cytoplasmic side of the transmembrane region (center right panel) shows a good fit between Sav1866 and the P-gp map. Asymmetry in this region in the P-gp map will arise from additional density that is likely to be due to the longer N-terminal region preceding

TM helix 1 as well as the long linker joining NBD1 with TMD2. The slice through the NBD region (bottom right panel) also shows a good overall fit between the map and the Sav1866 structure. Arrows 12a and 12b indicate regions of asymmetry in the P-gp map corresponding to arrows 6a and 6b in panel (a).

The overall impression from this comparison is that Sav1866 probably represents a reasonably good starting model for P-gp in the presence of AMP-PNP. There are differences in the precise trajectories of transmembrane helical elements, but this is to be expected given the low sequence identity between the two proteins in the transmembrane region (~20%). A model of P-gp based on Sav1866 but using "adjustments" or "constraints" from the EM-derived map would therefore be informative.

1.3.4
Drug Binding Sites in P-Glycoprotein

The involvement of P-gp in the multidrug resistance phenotype has prompted extensive study to uncover the mechanism of this polyspecificity. Two possibilities are most likely: (i) the presence of a single domain to which drugs display a loose association and (ii) multiple drug binding sites, each with a defined specificity.

The former option was proposed initially to take into account the nuances of P-gp-mediated transport. It was suggested [178] that P-gp behaved as a "flippase." The model suggested that the protein extracted drugs directly from the lipid milieu rather than the conventional wisdom of entering the translocation machinery through the cytoplasm. This suggestion was supported by the drug transport studies using acetoxymethyl esters (AM) of fluorescent probes (e.g., calcein-AM) [179]. The AM derivatives were nonfluorescent substrates for P-gp transport and readily converted to the fluorescent, nontransported compound by cytoplasmic esterases. Cells containing P-gp did not display any fluorescence although it rapidly appeared following P-gp inhibition. The results indicated that the drugs were expelled from the cell prior to reaching the cytoplasm. Further support was provided by Raviv *et al.* who demonstrated that photocross-linking of the membrane localized probe [^{125}I]-INA to P-gp through direct energy transfer from the substrates rhodamine 123 or doxorubicin [180]. Another key premise of the "flippase" model suggested that the "flip-flop" of drugs across the lipid bilayer was a slow process, yet this clearly was not the case for a number of substrates and modulators of P-gp [181, 182]. Finally, it was suggested by this model that drugs interact with the protein on a hydrophobic interface, rather than via a "classical" binding site. As discussed below and in subsequent sections of this chapter, there are clearly defined and specific sites for drug interaction with P-gp. Although two tenets of the flippase model are flawed, the suggestion that drug extraction occurs directly from the lipid bilayer is an accepted and characteristic feature of P-gp-mediated transport.

The quest to determine whether P-gp contains multiple drug binding sites employed a number of distinct approaches. For example, site-directed mutagenesis revealed a multitude of residues that when mutated could alter the pattern of drug resistance conferred by P-gp [183–185]. The effects of mutations on the activity were

analyzed by cytotoxicity assays. However, this strategy could not attribute the residues directly to the drug binding event, and the mutations could conceivably interfere at any stage of the translocation process. In contrast, a number of investigators employed a direct approach to the issue of the drug–P-gp interaction. Early studies employing the covalent labeling of P-gp with photolabile derivatives of recognized substrates and subsequent proteolytic cleavage suggested that both halves of the protein contributed to binding [186–189]. However, this does not preclude the possibility that both halves contribute to a single site for drug binding. In addition, the flexibility and high reactivity of photoactivated compounds generate a high degree of nonspecific covalent attachment [190]. The best photoaffinity labeling evidence for the presence of multiple drug recognition sites was obtained by Dey *et al.* [52] while examining the effects of the inhibitor *cis*-flupentixol on [^{125}I]-IAAP binding. P-gp labeled with [^{125}I]-IAAP and subjected to tryptic cleavage produced two polypeptides, both of which contained covalently attached [^{125}I]-IAAP. Addition of *cis*-flupentixol caused an increase in the affinity of [^{125}I]-IAAP binding at the C-terminal site. However, there was no effect of the inhibitor on [^{125}I]-IAAP binding to the N-terminal site. This finding demonstrates the presence of nonidentical sites for drug interaction on P-gp, and that for a subset of drugs, there are overlapping specificities at these sites.

The first account of noncompetitive drug interactions on P-gp was provided by Tamai and Safa using a radioligand binding assay with vinblastine and [^{3}H]-azidopine [191]. The term "competitive inhibition" is often misused in biochemistry and in its purest definition proves interaction at a common site. Consequently, the noncompetitive displacement of [^{3}H]-azidopine binding by vinblastine demonstrated that these two compounds interact at pharmacologically distinct regions. In contrast, the interaction between [^{3}H]-vinblastine and cyclosporin A was competitive [191]. These two investigations demonstrated not only the presence of multiple binding sites but also that these sites could interact with more than one compound. The presence of multiple drug interaction sites was confirmed by a number of groups, and the use of kinetic binding studies indicated that these sites were linked by a negative heterotropic allostery [192–194]. In their most comprehensive study, Martin *et al.* outlined the presence of at least four binding sites [60]. Some of the sites bound transported compounds only, while others were exclusive to modulators. However, this type of classification was achieved with only a small number of compounds compared to the spectrum of drugs recognized by P-gp. Although the interactions described by these investigations do reveal pharmacologically distinct drug binding sites, they do not inform on their precise locations or their spatial proximities.

Residues putatively involved in the drug recognition process have therefore been identified via a combination of direct photolabeling, cysteine scanning mutagenesis, and chemical cross-linking (Table 1.1).

These techniques allow accessibility of residues in P-gp TM helices to be mapped by covalent attachment of substrates. By their nature, these studies are not unbiased. Photolabeling with propafenones identifies accessible methionine residues that are presumed to be at or near the drug binding sites, and cysteine scanning mutagenesis also has an inherent bias. However, these techniques remain the closest we have to definitive identification of the amino acids composing the drug binding sites. A

Table 1.1 Residues implicated in the substrate binding pocket(s) of P-glycoprotein.

TM	Residue	Label	Notes/effect	References
1	65*	MTS-rhodamine	200% Vpl-stimulated ATPase activity after MTS-rhodamine labeling	[55, 56]
		MTS-verapamil	Persistent stimulation after labeling. No further stimulation seen with other drugs suggesting 100% labeling or that the MTS-Vpl blocks other drug binding. CsA and Vpl pretreatment inhibits labeling	[195]
2	118, 125	MTS-verapamil	Persistent stimulation by MTS-Vpl, not inhibited by pretreatment with Vpl	[53, 54, 196]
3	197*	Propafenone	Major photolabeled residue from peptide mass fingerprinting	[197]
4	222*	Dibromobimane	>50% inhibition of ATPase activity. Could be rescued by preincubation with Vpl, Vbl, and Col	[196]
		MTS-verapamil	ATPase activity inhibited by reaction with MTS-Vpl and restored by prior incubation with Vpl	[53, 54]
5	306*	MTS-verapamil	Persistent stimulation with MTS-Vpl. Prior incubation with Vpl inhibits labeling	[198]
	311*	MTS reagents	Reaction of 306C with MTSET and MTSES alters the potency of Vpl stimulation	[199]
		Propafenone	Major photolabeled residue from peptide mass fingerprinting	[197]
6	331	Maleimides	CM accessible all states, BM AMP-PNP	[200]
	333	Maleimides	CM accessible all states, BM Vi-trapped	[53, 54]
	335	Maleimides	CM accessible all states	[201]
	337	Maleimides	CM accessible all states, BM Vi-trapped	
	339	Maleimides	CM accessible all states, BM Vi-trapped and Apo	
	339	Dibromobimane	>50% inhibition of ATPase. Could be rescued by preincubation with Vpl, Vbl, and Col	

(*Continued*)

Table 1.1 (*Continued*)

TM	Residue	Label	Notes/effect	References
	341	Maleimides	CM accessible all states	
	342[1]	Dibromobimane	>50% inhibition of ATPase. Could be rescued by preincubation with Vpl	
	342*	MTS-verapamil	ATPase activity inhibited by reaction with MTS-Vpl and restored by prior incubation with Vpl	
	343	Maleimides	CM, BM, and FM accessible all states	
7	728*	MTS-verapamil	Persistent stimulation. No further stimulation seen with other drugs. Reduction of this stimulation by cyclosporin A and pretreatment with Vpl	[202]
	725, 729		Persistent stimulation by MTS-Vpl, not inhibited by pretreatment with Vpl	
8	766	MTS-verapamil	Persistent stimulation by MTS-Vpl, not inhibited by pretreatment with Vpl	[196]
	769*	Propafenone	Major photolabeled residue from peptide mass fingerprinting	[197]
9	841, 842	MTS-verapamil	Persistent stimulation by MTS-Vpl, not inhibited by pretreatment with Vpl	[53, 54]
10	868*, 872*	Dibromobimane	>50% inhibition of ATPase activity. Could be rescued by preincubation with Vpl, Vbl, and Col	[196]
	871	MTS-verapamil	Persistent stimulation by MTS-Vpl, not inhibited by pretreatment with Vpl	[53, 54]
11	942*, 945*	Dibromobimane	>50% inhibition of ATPase activity. Could be rescued by preincubation with Vpl, Vbl, and Col	[203]
	951*	Propafenone	Major photolabeled residue from peptide mass fingerprinting	[197]
12	975*[1,2,3], 982[2,3], 985[1]	Dibromobimane	50% inhibition of ATPase activity. Could be rescued by preincubation with Vpl[1], Vbl[2], and Col[3] (verapamil, vinblastine, and colchicine respectively)	[200]
	984*	MTS-verapamil	ATPase activity inhibited by reaction with MTS-Vpl and restored by prior incubation with Vpl	[53, 54]

number of substrates have been used for photolabeling of P-gp to define regions of the protein that play a role in solute binding and transport. Initially, studies with photoactive P-gp ligands were able to ascribe drug binding sites only to the C-terminal ends of the 6th and 12th TM α-helices [189, 204] (Table 1.2).

However, recent advances in trypsin cleavage and subsequent mass spectrometric identification of peptide fragments, coupled with the development of photoactive derivatives of propafenones, have enabled Chiba and colleagues to identify specific amino acids labeled during cross-linking [197]. Quantitative analysis of photolabeling indicates major sites for reactivity within TM3 (methionine 197), TM5 (methionine 311), TM8 (methionine 769), and TM11 (methionine 951) (see Table 1.1 and Ref. [197]). Minor peaks were identified within several other TM α-helices including TM1 and 12 [197] (Table 1.2).

Rather than rely on endogenous residues for cross-linking, the groups of Clarke, and to a lesser extent that of Callaghan, employed directed cysteine mutagenesis and chemical labeling to determine residue accessibility in the TM regions. Both sets of studies relied on the generation of functional cysteine-less versions of P-gp [212, 213]. The ATPase activity of this cysteine-less isoform was stimulated by the cross-linker dibromobimane (Dbbr), and this property was employed by Loo and Clarke to identify amino acids that when mutated to cysteine and then derivatized by Dbbr showed inhibited ATPase activity that could be prevented by prior incubation with another drug substrate (Table 1.1) [31, 196, 200]. Such residues are proposed to be part of the drug binding site for the "protective" drug. Similarly, the synthesis of sulfhydryl reactive rhodamine and verapamil analogues (methanethiosulfonates, MTS), which caused persistent stimulation of ATPase activity, allowed the identification of residues presumed to be involved in rhodamine or verapamil binding [53–56, 195, 198, 199, 202]. In an alternative approach, the Callaghan group introduced cysteine residues in a cysteine-free version of P-gp [213] and investigated their accessibility in distinct states of the catalytic cycle to maleimides of differing physicochemical properties (Table 1.1) [201, 214]. Residue 339 in TM6 appears to be of particular interest as conflicting results have been obtained. C339 (residue 339 mutated to cysteine) is labeled by maleimides with subsequent effect on the ATPase activity of P-gp. However, this is not a consequence of altered drug binding capacity suggesting that 339 is not a direct contributor of the drug binding pocket [214]. In contrast, C339 is labeled by Dbbr with concomitant inhibition of ATPase activity, which is rescued by preincubation with verapamil, vinblastine, and colchicine [200]. This discrepancy is a reflection of the different criteria used to identify a residue involved in drug binding – one of which is an indirect measure of drug binding.

1.3.5
Structural Interpretation of Drug Binding

Are the data in Tables 1.1 and 1.2 of sufficient consistency to predict where drug binding sites might reside on a structural model of P-gp? To answer this question, we have assigned two levels of certainty to a residue being directly implicated in drug binding. In the first category, we place those residues that are heavily labeled in

Table 1.2 Additional localization studies of the drug binding regions on P-glycoprotein.

TM	Residue	Notes/effect	References
1	61, 64	Residues around one-helical turn of TM1 mutated and alteration to the profile of drug resistance determined	[205, 206]
1	68, 69	Few major peaks of labeling with propafenone derivatives	[197]
ECL1	105–111	Few major peaks of labeling with propafenone derivatives	[197]
ICD1	185	Gly to Val at residue 185 of P-gp alters drug resistance and photolabeling profiles	[204]
6	338, 339	P-gp mutants isolated from drug-resistant cell lines. Photolabeling with IAAP that could no longer be inhibited by CsA. A quadruple mutant with residues in TM9 below showed no photolabeling of IAAP	[207, 208]
6-NBD1	358–456	Photolabeling with IAAP; trypsin and chymotrypsin digest and immunoprecipitation	[189]
ICD1	791–796	Secondary peaks of labeling with propafenone derivatives	[197]
9	837, 839	P-gp mutants isolated from drug resistant cell lines. A quadruple mutant together with TM6 mutants above showed no photolabeling of IAAP	[207, 208]
11	948, 949	Secondary peaks of labeling with propafenone derivatives	[197]
	949, 953	Scanning alanine mutagenesis investigating effects at the level of drug transport	[209]
12	969	Secondary peaks of labeling with propafenone derivatives	[197]
	975, 981, 983	Alanine scanning mutagenesis of TM12 and effects on IAAP transport	[210]
11–12	953–1007	Photolabeling of P-gp with a derivative of CsA, combined with purification, digest, and chemical mapping	[211]
12-NBD2	979–1048	Photolabeling with IAAP; trypsin and chymotrypsin digest and immunoprecipitation	[189]

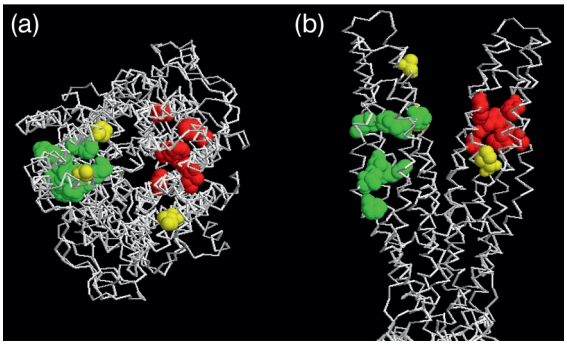

Figure 1.8 Drug binding site localization data in P-gp. Residues (in green, red, and yellow) that are interpreted as contributing to drug binding in P-gp (see Table 1.1 and text) are mapped onto the crystal structure of Sav1866. Residues cluster into two groups: those in green form a slide of residues primarily on the lipid-exposed surface of the molecule (right-hand panel) and those in red form a pocket open to the lumen of the structure. Three residues (yellow) do not fit this pattern.

propafenone photolabeling studies and residues that when altered to cysteine and derivatized result in altered ATPase activity that can be rescued by preincubation with drugs (Table 1.1, indicated by an asterisk in the second column). Other residues listed in Table 1.1 and all those in Table 1.2 are considered to be more indirect measures of drug binding site identification. These former residues have been mapped to their equivalents in Sav1866 [90] and are shown spatially in Figure 1.8.

The residues classified as robust indicators of substrate binding show two obvious clusters on opposite wings of the Sav1866 dimer, with each site primarily contributed by one homologous half. In P-gp this would translate as the first site being exposed to the lumen of the protein, contributed by amino acids at the approximate centers of TM3–6, and a small contribution from TM7 (red, Figure 1.8a). The second site, rather than being a cluster of amino acids, is better portrayed as a slide of residues along the lipid-exposed surface of TM10–12 (green, Figure 1.8b). Whether one of these sites (site 2) represents a hydrophobic interaction site for lipophilic drugs, and the other (site 1) represents a modifying site for hydrophilic ligands, is open to speculation.

1.3.6 Inhibitors of P-gp

The earliest investigations into the resistant phenotype in cancer revealed that cells displayed reduced sensitivity to a large number of anticancer drugs [215, 216]. Moreover, the compounds were often unrelated chemically or functionally. This suggested the presence of multiple mechanisms or perhaps a promiscuous contributing factor. In the case of P-glycoprotein, the latter is certainly true. So far, it has been established that P-gp is capable of interacting with over 200 compounds that may be classed as transported substrates or nontransported modulators [217]. The broad spectrum of resistance and the poor prognosis for patients necessitated strategies to circumvent the actions of P-gp. As a result, a great deal of effort has been directed toward the development of chemical inhibitors of P-gp. During the early 1980s, the

emerging broad spectrum of possible inhibitors was greeted with a certain degree of optimism since established, clinically used, compounds could restore some sensitivity to chemotherapy, thereby bypassing the tortuous pathway of preclinical drug development. This use of established drugs for the modulation of P-gp function formed the first generation of inhibitors [218–223]. The strategy was underpinned by numerous biochemical investigations that revealed the successful inhibition of P-gp *in vitro* using a wide range of approaches including cytotoxicity assessment, whole-cell accumulation, and modulation of ATP hydrolysis. The calcium channel blocker verapamil took the most rapid early ascent from such *in vitro* assays to clinical trials [224, 225]. Unfortunately, achieving significant inhibition of P-gp function required plasma concentrations that were considerably higher than those needed for calcium channel blockade. As a result, verapamil treatment was associated with nonspecific toxicity in patients [224, 225]. A similar situation was observed for many first-generation compounds including the immunosuppressant cyclosporin A [226, 227]. In hindsight, the fact that *in vitro* potencies of the drugs rarely reached submicromolar concentrations foreshadowed the lack of effectiveness of their use *in vivo*.

The second-generation P-gp inhibitor emerged directly from the previous generation and involved the use of chemical derivatives of the drugs with a view to eliciting less nonspecific toxicity. For example, the D-isomer of verapamil produced considerably lower calcium channel blockade than the L-isomer, whereas both forms produced equivalent inhibition of P-gp [228]. Therefore, the use of D-verapamil was proposed to raise the tolerated dosage of the drug to levels in the plasma that enabled efficient inhibition of P-gp. Overall, the strategy did achieve lower (but not negligible) levels of toxicity due to D-verapamil; however, there were reports of increased side effects of anticancer drugs [229, 230]. As a result, the increased anticancer drug toxicity required dose reduction of chemotherapy, thereby negating any positive effects generated by the inhibition of P-gp activity. The nonimmunosuppresive cyclosporin A (CsA) derivative PSC833 (valspodar) also offered the potential to reach sufficiently high plasma concentrations to affect P-gp inhibition [231]. Unlike CsA, valspodar displayed little inherent toxicity [232]; however, its addition caused a reduced clearance and metabolism of anticancer drugs [232, 233]. The reason for this effect was the competitive inhibition of anticancer drug metabolism by CYP3A isoforms, thereby prolonging drug residence in the plasma and increasing nonspecific toxicity [232, 233]. However, it was suggested that restricting the bioavailability of valspodar and modification in the dosage of anticancer drugs could overcome these effects and warranted further clinical assessment. Two recent phase III trials reported pharmacokinetic interactions that were overcome; however, the combined treatment with valspodar and anticancer drug did not positively impact patient survival [234].

The problems associated with the first two generations of P-gp inhibitors necessitated an alternative approach to P-gp inhibition. This was achieved through the use of combinatorial chemistry to discover novel classes of lead compounds. The two most notable success stories of this third-generation P-gp inhibitor were GF120918 (elacridar) [235] and XR9576 (tariquidar) [236]. Both drugs were characterized by nanomolar potencies for interaction with P-gp resulting in a high degree of optimism. This was further enhanced by *in vivo* studies demonstrating that the phar-

macokinetic interaction between anticancer drugs and these two modulators at the level of CYP3A metabolism was not as significant as with the earlier generations. Inherent toxicities are also less pronounced and both drugs remain in late-stage clinical trials. The only available data from phase III trials has been presented for tariquidar in combination with doxorubicin and taxane containing chemotherapy regimes in breast cancer [237]. The results indicated that only a small subset of patients exhibited benefit with combined administration of anticancer drug and tariquidar. Further clinical trials in a variety of cancer types and chemotherapy regimes are required to elucidate the true worth of tariquidar in the restoration of chemotherapy in resistant cancer.

In summary, over 30 years of research into P-gp inhibitors has generated only a handful of clinically usable compounds. Clearly, there is a pressing need for development of new inhibitors. The use of more rational or directed drug development has not yet been exploited and thus the provision of structural information on the drug binding sites of P-gp will prove instrumental.

1.3.7
What Properties Are Shared by Drugs that Interact with P-Glycoprotein?

A number of attempts have been made to compare physicochemical properties of a large number of compounds capable of interacting with P-gp. This comparative analysis would ideally generate a set of pharmacophoric "rules" to facilitate design of potent inhibitors of P-gp. The earliest attempts [238, 239] failed to reveal any specific criteria; however, they did suggest that substrates and inhibitors shared the following physicochemical properties: (i) planar aromatic rings, (ii) a basic nitrogen atom, and (iii) lipophilicity. Subsequent functional studies [240] classified P-gp modulators and substrates into distinct subsections, but this provided only weak discrimination between interacting drugs.

A comprehensive study by Seelig [241] was the first to produce a specific pharmacophoric pattern for recognition by P-gp and used a strategy involving examination of functional groups capable of hydrogen bonding to P-gp [241]. The strongest interacting compounds contained two or three electron donor groups, and moreover, these groups displayed fixed spatial separation (e.g., type I inhibitors have 2.5 ± 0.3 Å spacing between two e^- donor groups). The data were supported by the high degree of hydrogen bonding donor and acceptor moieties within the transmembrane helices of P-gp. A follow-up study [242] proposed that substrates and inhibitors varied in their propensity to form hydrogen bonds and that this was a key to defining the affinity for interaction. Inhibitors were proposed to display higher affinity due to stronger and more numerous hydrogen bonds with P-gp. This would result in slower dissociation rates for inhibitors, a property that was supported by radioligand binding studies with [^{3}H]-vinblastine (substrate) and [^{3}H]-XR9576 (inhibitor) [60].

During the past 5 years, an increasing number of more sophisticated bioinformatic or modeling approaches have been employed in the quest to generate a detailed map for the pharmacophore of P-gp substrates and inhibitors. The drug–P-gp interaction

is a complex one, and as result, these strategies have focused on single classes of compounds. Raad *et al*. used a 3D quantitative structure–activity (QSAR) analysis for natural and synthetic coumarin derivatives [243]. The electrostatic and steric volume factors provided the greatest predictive power in assessing potency of interaction with P-gp. Moreover, a neutral hydrophobic group on the C4 position of the coumarin group greatly affected potency. The nature of the aromatic ring substitution of propafenone derivatives also greatly influenced the affinity of this class of compounds. An e^- donating moiety was a positive factor, whereas a bulky substituent (e.g., diphenyalkamine) weakened the interaction [244]. Labrie *et al*. [245] confined their analysis to derivatives of the potent anthranilamide tariquidar and focused on four distinct chemical regions of the molecule [245]. Once again, the affinity of derivative interaction with P-gp was greatly influenced by steric effects, electrostatic potential, and positioning of the hydrophobic moieties.

Thus, it is clear that two decades of structure–activity analyses have generated only subtle alterations to the originally proposed pharmacophoric elements of drugs interacting with P-gp. What has emerged is that the interaction is clearly a complex one and highly specific at a local level, which is far removed from the earliest suggestions based only on hydrophobicity. In addition, there are subtle, but important, differences between classes of drugs interacting with P-gp as might be expected given that the protein has multiple distinct binding sites. Two recent investigations employing a larger number of compounds have revealed some more specific requirements [245, 246]. Affinity of the drug–P-gp interaction is proportional to the hydrogen bond strength and the specific distance between the hydrogen bonding groups. Substrates may be more hydrophilic than originally proposed, and in fact the K_m for transport does not correlate with log *P* values. The emerging picture from these investigations is that high-affinity compounds minimally contain (i) two hydrophobic groups separated by 16.5 Å and (ii) two hydrogen bond acceptor groups that are 11.5 Å apart. Fully exploiting these data will require data on the structure of drug binding sites and the molecular basis underlying the "polyspecificity" of P-gp.

1.3.8
Postscript: Further X-Ray Crystallographic Studies and a Structure for the Nucleotide-Free State of P-Glycoprotein

Just prior to the proof stage of this chapter, a report describing the structure of P-gp in the nucleotide-free (apo) state appeared from the group of Chang [247]. Since this happy event merits the short description below, it is also worth updating the chapter with very recent structural insights emerging for other ABC transporters. Two structures for entire bacterial ABC proteins have been added to the growing list: (a) The structure of the methionine importer MetNI, from the laboratory of Rees [248], displays a similar TMD fold to the ModBC and MalFGK2 structures. (b) A second structure for MalFGK2 in the absence of nucleotide and in an apparently inward-facing configuration [249] beautifully confirms the predictions of Davidson about the likely conformational changes in the bacterial importers which were based on comparison of the ModBC and (outward facing) MalFGK2 structures - see Figure 1.3.

Lastly, the P-gp structure: It is the first structure of a eukaryotic ABC protein where the resolution is sufficient to trace most of the (single) polypeptide chain. Moreover, Aller and his co-workers [247] were able to generate two further structures of the nucleotide-free protein with different chiral forms of a cyclic peptide inhibitor bound to the TMD portion of the protein. Although the structural biology group were unable to generate a structure for the nucleotide-bound form of the protein, the existence of a lower resolution EM map (Figure 1.6) and its strong similarity to the nucleotide-bound Sav1866 structure will allow us insight into the conformational changes undergone by P-gp when moving between nucleotide-bound and nucleotide-free states: It seems likely that transmembrane helices 4 and 5 in TMD1, and their equivalents in TMD2 (helices 10 and 11) rotate as pairs around hinge regions in the extracellular loops of P-gp giving a tweezers-like opening and closing of the TMDs. This motion is associated with a separation and coming together of the NBDs, mediated by the intracytoplasmic loops 2 and 4 connecting transmembrane helices 4 to 5 and 10 to 11 respectively. As predicted from the Sav1866 structure, these intracytoplasmic loops cross over to the opposite side of the molecule in P-gp.

1.4 Summary

Progress in the structural description of ABC transporters is beginning to reach the stage where clinically relevant outputs may arise. A combination of low- and high-resolution structural data, alongside molecular homology modeling, is giving us a much clearer picture of transporters such as P-gp. Compared to even 10 years ago, our knowledge has advanced enormously. Given a similar rate of progress, the probability of designing novel inhibitors and drugs with input from structural data seems high over the next decade. Knowledge of the structure of binding sites in P-gp will be vital in the design of new inhibitors of the protein.

References

1 Klein, I., Sarkadi, B., and Varadi, A. (1999) An inventory of the human ABC proteins. *Biochimica et Biophysica Acta*, **1461** (2), 237–262.

2 Linton, K.J. and Higgins, C.F. (1998) The *Escherichia coli* ATP-binding cassette (ABC) proteins. *Molecular Microbiology*, **28** (1), 5–13.

3 Rosenberg, M.F., Mao, Q., Holzenburg, A., Ford, R.C., Deeley, R.G., and Cole, S.P. (2001) The structure of the multidrug resistance protein 1 (MRP1/ABCC1). Crystallization and single-particle analysis. *The Journal of Biological Chemistry*, **276** (19), 16076–16082.

4 Higgins, C.F. (1992) ABC transporters: from microorganisms to man. *Annual Review of Cell Biology*, **8**, 67–113.

5 Childs, S. and Ling, V. (1994) The MDR superfamily of genes and its biological implications, in *Important Advances in Oncology* (eds V.T. DeVita, S. Hellman, and S.A. Rosenberg), Lippincott, Philadelphia, PA, pp. 21–36.

6 Dean, M. and Allikmets, R. (1995) Evolution of ATP-binding cassette

transporter genes. *Current Opinion in Genetics & Development*, **5** (6), 779–785.

7 Dean, M., Rzhetsky, A., and Allikmets, R. (2001) The human ATP-binding cassette (ABC) transporter superfamily. *Genome Research*, **11** (7), 1156–1166.

8 Mutch, D.M., Anderle, P., Fiaux, M., Mansourian, R., Vidal, K., Wahli, W., Williamson, G., and Roberts, M.A. (2004) Regional variations in ABC transporter expression along the mouse intestinal tract. *Physiological Genomics*, **17** (1), 11–20.

9 Kerr, I.D. (2002) Structure and association of ATP-binding cassette transporter nucleotide-binding domains. *Biochimica et Biophysica Acta*, **1561** (1), 47–64.

10 Hosie, A.H., Allaway, D., Jones, M.A., Walshaw, D.L., Johnston, A.W., and Poole, P.S. (2001) Solute-binding protein-dependent ABC transporters are responsible for solute efflux in addition to solute uptake. *Molecular Microbiology*, **40**, 1449–1459.

11 Kast, C., Canfield, V., Levenson, R., and Gros, P. (1995) Membrane topology of P-glycoprotein as determined by epitope insertion: transmembrane organization of the N-terminal domain of mdr3. *Biochemistry*, **34** (13), 4402–4411.

12 Kast, C., Canfield, V., Levenson, R., and Gros, P. (1996) Transmembrane organization of mouse P-glycoprotein determined by epitope insertion and immunofluorescence. *The Journal of Biological Chemistry*, **271** (16), 9240–9248.

13 Tusnady, G.E., Bakos, E., Varadi, A., and Sarkadi, B. (1997) Membrane topology distinguishes a subfamily of the ATP-binding cassette (ABC) transporters. *FEBS Letters*, **402** (1), 1–3.

14 Jha, S., Karnani, N., Dhar, S.K., Mukhopadhayay, K., Shukla, S., Saini, P., Mukhopadhayay, G., and Prasad, R. (2003) Purification and characterization of the N-terminal nucleotide binding domain of an ABC drug transporter of *Candida albicans*: uncommon cysteine 193 of Walker A is critical for ATP hydrolysis. *Biochemistry*, **42** (36), 10822–10832.

15 Locher, K.P. and Borths, E. (2004) ABC transporter architecture and mechanism: implications from the crystal structures of BtuCD and BtuF. *FEBS Letters*, **564** (3), 264–268.

16 Azzaria, M., Schurr, E., and Gros, P. (1989) Discrete mutations introduced in the predicted nucleotide-binding sites of the mdr1 gene abolish its ability to confer multidrug resistance. *Molecular and Cellular Biology*, **9** (12), 5289–5297.

17 Al-Shawi, M.K. and Senior, A.E. (1993) Characterization of the adenosine triphosphatase activity of Chinese hamster P-glycoprotein. *The Journal of Biological Chemistry*, **268** (6), 4197–4206.

18 Gao, M., Cui, H.R., Loe, D.W., Grant, C.E., Almquist, K.C., Cole, S.P., and Deeley, R.G. (2000) Comparison of the functional characteristics of the nucleotide binding domains of multidrug resistance protein 1. *The Journal of Biological Chemistry*, **275** (17), 13098–13108.

19 Hou, Y., Cui, L., Riordan, J.R., and Chang, X. (2000) Allosteric interactions between the two non-equivalent nucleotide binding domains of multidrug resistance protein MRP1. *The Journal of Biological Chemistry*, **275** (27), 20280–20287.

20 Lapinski, P.E., Neubig, R.R., and Raghavan, M. (2001) Walker A lysine mutations of TAP1 and TAP2 interfere with peptide translocation but not peptide binding. *The Journal of Biological Chemistry*, **276** (10), 7526–7533.

21 Saveanu, L., Daniel, S., and van Endert, P.M. (2001) Distinct functions of the ATP binding cassettes of transporters associated with antigen processing: a mutational analysis of Walker A and B sequences. *The Journal of Biological Chemistry*, **276** (25), 22107–22113.

22 Proff, C. and Kolling, R. (2001) Functional asymmetry of the two nucleotide binding domains in the ABC transporter Ste6. *Molecular & General Genetics*, **264** (6), 883–893.

23 Anderson, M.P. and Welsh, M.J. (1992) Regulation by ATP and ADP of CFTR

chloride channels that contain mutant nucleotide-binding domains. *Science*, **257** (5077), 1701–1704.

24 Carson, M.R., Travis, S.M., and Welsh, M.J. (1995) The two nucleotide-binding domains of cystic fibrosis transmembrane conductance regulator (CFTR) have distinct functions in controlling channel activity. *The Journal of Biological Chemistry*, **270** (4), 1711–1717.

25 Cui, L., Aleksandrov, L., Chang, X.B., Hou, Y.X., He, L., Hegedus, T., Gentzsch, M., Aleksandrov, A., Balch, W.E., and Riordan, J.R. (2007) Domain interdependence in the biosynthetic assembly of CFTR. *Journal of Molecular Biology*, **365**, 981–994.

26 Senior, A.E., al-Shawi, M.K., and Urbatsch, I.L. (1995) ATP hydrolysis by multidrug-resistance protein from Chinese hamster ovary cells. *Journal of Bioenergetics and Biomembranes*, **27** (1), 31–36.

27 Szakacs, G., Ozvegy, C., Bakos, E., Sarkadi, B., and Varadi, A. (2001) Role of glycine-534 and glycine-1179 of human multidrug resistance protein (MDR1) in drug-mediated control of ATP hydrolysis. *The Biochemical Journal*, **356** (Pt 1), 71–75.

28 Urbatsch, I.L., Gimi, K., Wilke-Mounts, S., and Senior, A.E. (2000) Conserved Walker A Ser residues in the catalytic sites of P-glycoprotein are critical for catalysis and involved primarily at the transition state step. *The Journal of Biological Chemistry*, **275** (32), 25031–25038.

29 Urbatsch, I.L., Gimi, K., Wilke-Mounts, S., and Senior, A.E. (2000) Investigation of the role of glutamine-471 and glutamine-1114 in the two catalytic sites of P-glycoprotein. *Biochemistry*, **39** (39), 11921–11927.

30 Takada, Y., Yamada, K., Taguchi, Y., Kino, K., Matsuo, M., Tucker, S.J., Komano, T., Amachi, T., and Ueda, K. (1998) Non-equivalent cooperation between the two nucleotide-binding folds of P-glycoprotein. *Biochimica et Biophysica Acta*, **1373** (1), 131–136.

31 Loo, T.W. and Clarke, D.M. (1995) Covalent modification of human P-glycoprotein mutants containing a single cysteine in either nucleotide-binding fold abolishes drug-stimulated ATPase activity. *The Journal of Biological Chemistry*, **270** (39), 22957–22961.

32 Hrycyna, C.A., Ramachandra, M., Germann, U.A., Cheng, P.W., Pastan, I., and Gottesman, M.M. (1999) Both ATP sites of human P-glycoprotein are essential but not symmetric. *Biochemistry*, **38** (42), 13887–13899.

33 Vigano, C., Julien, M., Carrier, I., Gros, P., and Ruysschaert, J.M. (2002) Structural and functional asymmetry of the nucleotide-binding domains of P-glycoprotein investigated by attenuated total reflection Fourier transform infrared spectroscopy. *The Journal of Biological Chemistry*, **277** (7), 5008–5016.

34 Berridge, G., Walker, J.A., Callaghan, R., and Kerr, I.D. (2003) The nucleotide-binding domains of P-glycoprotein. Functional symmetry in the isolated domain demonstrated by *N*-ethylmaleimide labelling. *European Journal of Biochemistry*, **270** (7), 1483–1492.

35 Walker, J.E., Saraste, M., Runswick, M.J., and Gay, N.J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *The EMBO Journal*, **1** (8), 945–951.

36 Young, J. and Holland, I.B. (1999) ABC transporters: bacterial exporters-revisited five years on. *Biochimica et Biophysica Acta*, **1461** (2), 177–200.

37 Diederichs, K., Diez, J., Greller, G., Muller, C., Breed, J., Schnell, C., Vonrhein, C., Boos, W., and Welte, W. (2000) Crystal structure of MalK, the ATPase subunit of the trehalose/maltose ABC transporter of the archaeon *Thermococcus litoralis*. *The EMBO Journal*, **19** (22), 5951–5961.

38 Ambudkar, S.V., Kim, I.W., Xia, D., and Sauna, Z.E. (2006) The A-loop, a novel

conserved aromatic acid subdomain upstream of the Walker A motif in ABC transporters, is critical for ATP binding. *FEBS Letters*, **580**, 1049–1055.

39 Holland, I.B. and Blight, M.A. (1999) ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. *Journal of Molecular Biology*, **293** (2), 381–399.

40 Martin, C., Berridge, G., Higgins, C.F., Mistry, P., Charlton, P., and Callaghan, R. (2000) Communication between multiple drug binding sites on P-glycoprotein. *Molecular Pharmacology*, **58**, 624–632.

41 Saurin, W., Hofnung, M., and Dassa, E. (1999) Getting in or out: early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters. *Journal of Molecular Evolution*, **48** (1), 22–41.

42 Locher, K.P., Lee, A.T., and Rees, D.C. (2002) The *E. coli* BtuCD structure: a framework for ABC transporter architecture and mechanism. *Science*, **296** (5570), 1091–1098.

43 Pinkett, H.W., Lee, A.T., Lum, P., Locher, K.P., and Rees, D.C. (2007) An inward-facing conformation of a putative metal-chelate-type ABC transporter. *Science*, **315**, 373–377.

44 Reyes, C.L. and Chang, G. (2005) Structure of the ABC transporter MsbA in complex with ADP·vanadate and lipopolysaccharide. *Science*, **308**, 1028–1031. Retraction in: Chang, G., Roth, C.B., Reyes, C.L., Pornillos, O., Chen, Y.J., and Chen, A.P. (2006) *Science*, **314**, 1875.

45 Chang, G. (2007) Retraction of "Structure of MsbA from *Vibrio cholera*: a multidrug resistance ABC transporter homolog in a closed conformation" [J. Mol. Biol. (2003) 330: 419–430]. *Journal of Molecular Biology*, **369**, 596.

46 Chang, G., Roth, C.B., Reyes, C.L., Pornillos, O., Chen, Y.J., and Chen, A.P. (2006) Retraction. *Science*, **314**, 1875.

47 Ward, A., Reyes, C.L., Yu, J., Roth, C.B., and Chang, G. (2007) Flexibility in the ABC transporter MsbA: alternating access with a twist. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 19005–19010.

48 Oldham, M.L., Khare, D., Quiocho, F.A., Davidson, A.L., and Chen, J. (2007) Crystal structure of a catalytic intermediate of the maltose transporter. *Nature*, **450**, 515–521.

49 Rosenberg, M.F., Callaghan, R., Ford, R.C., and Higgins, C.F. (1997) Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. *The Journal of Biological Chemistry*, **272** (16), 10685–10694.

50 Rosenberg, M.F., Velarde, G., Ford, R.C., Martin, C., Berridge, G., Kerr, I.D., Callaghan, R., Schmidlin, A., Wooding, C., Linton, K.J., and Higgins, C.F. (2001) Repacking of the transmembrane domains of P-glycoprotein during the transport ATPase cycle. *The EMBO Journal*, **20** (20), 5615–5625.

51 Rosenberg, M.F., Kamis, A.B., Aleksandrov, L.A., Ford, R.C., and Riordan, J.R. (2004) Purification and crystallization of the cystic fibrosis transmembrane conductance regulator (CFTR). *The Journal of Biological Chemistry*, **279**, 39051–39057.

52 Dey, S., Ramachandra, M., Pastan, I., Gottesman, M.M., and Ambudkar, S.V. (1997) Evidence for two nonidentical drug-interaction sites in the human P-glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America*, **94** (20), 10594–10599.

53 Loo, T.W. and Clarke, D.M. (2001) Defining the drug-binding site in the human multidrug resistance P-glycoprotein using a methanethiosulfonate analog of verapamil, MTS-verapamil. *The Journal of Biological Chemistry*, **276**, 14972–14979.

54 Loo, T.W. and Clarke, D.M. (2001) Determining the dimensions of the drug-

binding domain of human P-glycoprotein using thiol cross-linking compounds as molecular rulers. *The Journal of Biological Chemistry*, **276**, 36877–36880.

55 Loo, T.W. and Clarke, D.M. (2002) Vanadate trapping of nucleotide at the ATP-binding sites of human multidrug resistance P-glycoprotein exposes different residues to the drug-binding site. *Proceedings of the National Academy of Sciences of the United States of America*, **99** (6), 3511–3516.

56 Loo, T.W. and Clarke, D.M. (2002) Location of the rhodamine-binding site in the human multidrug resistance P-glycoprotein. *The Journal of Biological Chemistry*, **277** (46), 44332–44338.

57 Loo, T.W., Bartlett, M.C., and Clarke, D.M. (2003) Substrate-induced conformational changes in the transmembrane segments of human P-glycoprotein. Direct evidence for the substrate-induced fit mechanism for drug binding. *The Journal of Biological Chemistry*, **278** (16), 13603–13606.

58 Loo, T.W., Bartlett, M.C., and Clarke, D.M. (2003) Simultaneous binding of two different drugs in the binding pocket of the human multidrug resistance P-glycoprotein. *The Journal of Biological Chemistry*, **278** (41), 39706–39710.

59 Shapiro, A.B., Fox, K., Lam, P., and Ling, V. (1999) Stimulation of P-glycoprotein-mediated drug transport by prazosin and progesterone. Evidence for a third drug-binding site. *European Journal of Biochemistry*, **259**, 841–850.

60 Martin, C., Berridge, G., Mistry, P., Higgins, C., Charlton, P., and Callaghan, R. (1999) The molecular interaction of the high affinity reversal agent XR9576 with P-glycoprotein. *British Journal of Pharmacology*, **128** (2), 403–411.

61 Schmitt, L. and Tampe, R. (2002) Structure and mechanism of ABC transporters. *Current Opinion in Structural Biology*, **12** (6), 754–760.

62 Hung, L.W., Wang, I.X., Nikaido, K., Liu, P.Q., Ames, G.F., and Kim, S.H. (1998) Crystal structure of the ATP-binding subunit of an ABC transporter. *Nature*, **396** (6712), 703–707.

63 Hopfner, K.P., Karcher, A., Shin, D.S., Craig, L., Arthur, L.M., Carney, J.P., and Tainer, J.A. (2000) Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. *Cell*, **101** (7), 789–800.

64 Gadsby, D.C., Vergani, P., and Csanady, L. (2006) The ABC protein turned chloride channel whose failure causes cystic fibrosis. *Nature*, **440**, 477–483.

65 Moody, J.E., Millen, L., Binns, D., Hunt, J.F., and Thomas, P.J. (2002) Cooperative, ATP-dependent association of the nucleotide binding cassettes during the catalytic cycle of ATP-binding cassette transporters. *The Journal of Biological Chemistry*, **277** (24), 21111–21114.

66 Qian, Y.M., Qiu, W., Gao, M., Westlake, C.J., Cole, S.P., and Deeley, R.G. (2001) Characterization of binding of leukotriene C4 by human multidrug resistance protein 1: evidence of differential interactions with NH_2- and COOH-proximal halves of the protein. *The Journal of Biological Chemistry*, **276** (42), 38636–38644.

67 Hou, Y.X., Cui, L., Riordan, J.R., and Chang, X.B. (2002) ATP binding to the first nucleotide-binding domain of multidrug resistance protein MRP1 increases binding and hydrolysis of ATP and trapping of ADP at the second domain. *The Journal of Biological Chemistry*, **277** (7), 5110–5119.

68 Dousmanis, A.G., Nairn, A.C., and Gadsby, D.C. (2002) Distinct Mg^{2+}-dependent steps rate limit opening and closing of a single CFTR Cl^- channel. *The Journal of General Physiology*, **119** (6), 545–559.

69 Aleksandrov, L., Aleksandrov, A.A., Chang, X.B., and Riordan, J.R. (2002) The first nucleotide binding domain of cystic fibrosis transmembrane conductance regulator is a site of stable nucleotide interaction, whereas the second is a site of

rapid turnover. *The Journal of Biological Chemistry*, **277** (18), 15419–15425.

70 van Veen, H.W., Margolles, A., Muller, M., Higgins, C.F., and Konings, W.N. (2000) The homodimeric ATP-binding cassette transporter LmrA mediates multidrug transport by an alternating two-site (two-cylinder engine) mechanism. *The EMBO Journal*, **19** (11), 2503–2514.

71 van Veen, H.W., Margolles, A., Putman, M., Sakamoto, K., and Konings, W.N. (1999) Multidrug resistance in lactic acid bacteria: molecular mechanisms and clinical relevance. *Antonie van Leeuwenhoek*, **76** (1–4), 347–352.

72 van Veen, H.W., Putman, M., Margolles, A., Sakamoto, K., and Konings, W.N. (1999) Structure–function analysis of multidrug transporters in *Lactococcus lactis*. *Biochimica et Biophysica Acta*, **1461** (2), 201–206.

73 van Veen, H.W. and Konings, W.N. (1996) Multidrug resistance mediated by a bacterial homolog of the human multidrug transporter MDR1. *Proceedings of the National Academy of Sciences of the United States of America*, **93** (20), 10668–10672.

74 Shepard, R.L., Winter, M.A., Hsaio, S.C., Pearce, H.L., Beck, W.T., and Dantzig, A.H. (1998) Effect of modulators on the ATPase activity and vanadate nucleotide trapping of human P-glycoprotein. *Biochemical Pharmacology*, **56** (6), 719–727.

75 Sauna, Z.E. and Ambudkar, S.V. (2000) Evidence for a requirement for ATP hydrolysis at two distinct steps during a single turnover of the catalytic cycle of human P-glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America*, **97** (6), 2515–2520.

76 Kerr, K.M., Sauna, Z.E., and Ambudkar, S.V. (2001) Correlation between steady-state ATP hydrolysis and vanadate-induced ADP trapping in human P-glycoprotein. Evidence for ADP release as the rate-limiting step in the catalytic cycle and its modulation by substrates. *The Journal of Biological Chemistry*, **276** (12), 8657–8664.

77 Sauna, Z.E. and Ambudkar, S.V. (2001) Characterization of the catalytic cycle of ATP hydrolysis by human P-glycoprotein. The two ATP hydrolysis events in a single catalytic cycle are kinetically similar but affect different functional outcomes. *The Journal of Biological Chemistry*, **276** (15), 11653–11661.

78 Sauna, Z.E., Smith, M.M., Muller, M., and Ambudkar, S.V. (2001) Functionally similar vanadate-induced 8-azidoadenosine 5′-[alpha-^{32}P] diphosphate-trapped transition state intermediates of human P-glycoprotein are generated in the absence and presence of ATP hydrolysis. *The Journal of Biological Chemistry*, **276** (24), 21199–21208.

79 Sauna, Z.E., Smith, M.M., Muller, M., and Ambudkar, S.V. (2001) Evidence for the vectorial nature of drug (substrate)-stimulated ATP hydrolysis by human P-glycoprotein. *The Journal of Biological Chemistry*, **276** (36), 33301–33304.

80 Martin, C., Higgins, C.F., and Callaghan, R. (2001) The vinblastine binding site adopts high- and low-affinity conformations during a transport cycle of P-glycoprotein. *Biochemistry*, **40** (51), 15733–15742.

81 Davidson, A.L. and Chen, J. (2004) ATP-binding cassette transporters in bacteria. *Annual Review of Biochemistry*, **73**, 241–268.

82 Borges-Walmsley, M.I., McKeegan, K.S., and Walmsley, A.R. (2003) Structure and function of efflux pumps that confer resistance to drugs. *The Biochemical Journal*, **376** (Pt 2), 313–338.

83 Loo, T.W., Bartlett, M.C., and Clarke, D.M. (2002) The "LSGGQ" motif in each nucleotide-binding domain of human P-glycoprotein is adjacent to the opposing Walker A sequence. *The Journal of Biological Chemistry*, **277** (44), 41303–41306.

84 Loo, T.W., Bartlett, M.C., and Clarke, D.M. (2003) Drug binding in human P-glycoprotein causes conformational

changes in both nucleotide-binding domains. *The Journal of Biological Chemistry*, **278** (3), 1575–1578.

85 Callaghan, R., Ford, R.C., and Kerr, I.D. (2006) The translocation mechanism of P-glycoprotein. *FEBS Letters*, **580**, 1056–1063.

86 Schneider, E. and Hunke, S. (1998) ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS Microbiology Reviews*, **22** (1), 1–20.

87 Yuan, Y.R., Blecker, S., Martsinkevich, O., Millen, L., Thomas, P.J., and Hunt, J.F. (2001) The crystal structure of the MJ0796 ATP-binding cassette. Implications for the structural consequences of ATP hydrolysis in the active site of an ABC transporter. *The Journal of Biological Chemistry*, **276** (34), 32313–32321.

88 Chen, H.L., Gabrilovich, D., Tampe, R., Girgis, K.R., Nadaf, S., and Carbone, D.P. (1996) A functionally defective allele of TAP1 results in loss of MHC class I antigen presentation in a human lung cancer. *Nature Genetics*, **13** (2), 210–213.

89 Riordan, J.R. (1993) The cystic fibrosis transmembrane conductance regulator. *Annual Review of Physiology*, **55**, 609–630.

90 Dawson, R.J. and Locher, K.P. (2006) Structure of a bacterial multidrug ABC transporter. *Nature*, **443**, 180–185.

91 Murakami, S., Nakashima, R., Yamashita, E., and Yamaguchi, A. (2002) Crystal structure of bacterial multidrug efflux transporter AcrB. *Nature*, **419** (6907), 587–593.

92 Koronakis, V., Sharff, A., Koronakis, E., Luisi, B., and Hughes, C. (2000) Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature*, **405** (6789), 914–919.

93 Chang, G. (2003) Structure of MsbA from *Vibrio cholera*: a multidrug resistance ABC transporter homolog in a closed conformation. *Journal of Molecular Biology*, **330** (2), 419–430.

94 Chang, G. and Roth, C.B. (2001) Structure of MsbA from *E. coli*: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. *Science*, **293** (5536), 1793–1800.

95 Faham, S. and Bowie, J.U. (2002) Bicelle crystallization: a new method for crystallizing membrane proteins yields a monomeric bacteriorhodopsin structure. *Journal of Molecular Biology*, **316** (1), 1–6.

96 Nollert, P., Navarro, J., and Landau, E.M. (2002) Crystallization of membrane proteins in cubo. *Methods in Enzymology*, **343**, 183–199.

97 Armstrong, S., Tabernero, L., Zhang, H., Hermodsen, M., and Stauffacher, C. (1998) Powering the ABC transporters: the 2.5 Å crystal structure of the ABC domain of RbsA. *Pediatric Pulmonology*, **17**, 91–92.

98 Karpowich, N., Martsinkevich, O., Millen, L., Yuan, Y.R., Dai, P.L., MacVey, K., Thomas, P.J., and Hunt, J.F. (2001) Crystal structures of the MJ1267 ATP binding cassette reveal an induced-fit effect at the ATPase active site of an ABC transporter. *Structure*, **9** (7), 571–586.

99 Lowe, J., Cordell, S.C., and van den Ent, F. (2001) Crystal structure of the SMC head domain: an ABC ATPase with 900 residues antiparallel coiled-coil inserted. *Journal of Molecular Biology*, **306** (1), 25–35.

100 Lamers, M.H., Perrakis, A., Enzlin, J.H., Winterwerp, H.H., de Wind, N., and Sixma, T.K. (2000) The crystal structure of DNA mismatch repair protein MutS binding to a G × T mismatch. *Nature*, **407** (6805), 711–717.

101 Obmolova, G., Ban, C., Hsieh, P., and Yang, W. (2000) Crystal structures of mismatch repair protein MutS and its complex with a substrate DNA. *Nature*, **407** (6805), 703–710.

102 Gaudet, R. and Wiley, D.C. (2001) Structure of the ABC ATPase domain of human TAP1, the transporter associated with antigen processing. *The EMBO Journal*, **20** (17), 4964–4972.

103 Verdon, G., Albers, S.V., Dijkstra, B.W., Driessen, A.J., and Thunnissen, A.M. (2003) Crystal structures of the ATPase subunit of the glucose ABC transporter from *Sulfolobus solfataricus*: nucleotide-free and nucleotide-bound conformations. *Journal of Molecular Biology*, **330** (2), 343–358.

104 Schmitt, L., Benabdelhak, H., Blight, M.A., Holland, I.B., and Stubbs, M.T. (2003) Crystal structure of the nucleotide-binding domain of the ABC-transporter haemolysin B: identification of a variable region within ABC helical domains. *Journal of Molecular Biology*, **330** (2), 333–342.

105 Lewis, H.A., Buchanan, S.G., Burley, S.K., Conners, K., Dickey, M., Dorwart, M., Fowler, R., Gao, X., Guggino, W.B., Hendrickson, W.A., Hunt, J.F., Kearins, M.C., Lorimer, D., Maloney, P.C., Post, K.W., Rajashankar, K.R., Rutter, M.E., Sauder, J.M., Shriver, S., Thibodeau, P.H., Thomas, P.J., Zhang, M., Zhao, X., and Emtage, S. (2004) Structure of nucleotide-binding domain 1 of the cystic fibrosis transmembrane conductance regulator. *The EMBO Journal*, **23** (2), 282–293.

106 McKeegan, K.S., Borges-Walmsley, M.I., and Walmsley, A.R. (2003) The structure and function of drug pumps: an update. *Trends in Microbiology*, **11** (1), 21–29.

107 Davidson, A.L., Laghaeian, S.S., and Mannering, D.E. (1996) The maltose transport system of *Escherichia coli* displays positive cooperativity in ATP hydrolysis. *The Journal of Biological Chemistry*, **271** (9), 4858–4863.

108 Liu, C.E., Liu, P.Q., and Ames, G.F. (1997) Characterization of the adenosine triphosphatase activity of the periplasmic histidine permease, a traffic ATPase (ABC transporter). *The Journal of Biological Chemistry*, **272** (35), 21883–21891.

109 Stenham, D.R., Campbell, J.D., Sansom, M.S., Higgins, C.F., Kerr, I.D., and Linton, K.J. (2003) An atomic detail model for the human ATP binding cassette transporter P-glycoprotein derived from disulfide cross-linking and homology modelling. *The FASEB Journal*, **17** (15), 2287–2289.

110 Rosenberg, M.F., Callaghan, R., Modok, S., Higgins, C.F., and Ford, R.C. (2005) 3-D structure of P-glycoprotein: the transmembrane regions adopt an asymmetric configuration in the nucleotide-bound state. *The Journal of Biological Chemistry*, **280**, 2857–2862.

111 Hollenstein, K., Frei, D.C., and Locher, K.P. (2007) Structure of an ABC transporter in complex with its binding protein. *Nature*, **446**, 213–216.

112 McDevitt, C.A., Collins, R.F., Conway, M., Modok, S., Storm, J., Kerr, I.D., Ford, R.C., and Callaghan, R. (2006) Purification and 3D structural analysis of oligomeric human multidrug transporter ABCG2. *Structure*, **11**, 1623–1632.

113 Awayn, N.H., Rosenberg, M.F., Kamis, A.B., Aleksandrov, L.A., Riordan, J.R., and Ford, R.C. (2005) Crystallographic and single-particle analyses of native- and nucleotide-bound forms of the cystic fibrosis transmembrane conductance regulator (CFTR) protein. *Biochemical Society Transactions*, **33**, 996–999.

114 Rosenberg, M.F., Kamis, A.B., Callaghan, R., Higgins, C.F., and Ford, R.C. (2003) Three-dimensional structures of the mammalian multidrug resistance P-glycoprotein demonstrate major conformational changes in the transmembrane domains upon nucleotide binding. *The Journal of Biological Chemistry*, **278**, 8294–8299.

115 Chami, M., Steinfels, E., Orelle, C., Jault, J.M., Di Pietro, A., Rigaud, J.L., and Marco, S. (2002) Three-dimensional structure by cryo-electron microscopy of YvcC, an homodimeric ATP-binding cassette transporter from *Bacillus subtilis*. *Journal of Molecular Biology*, **315** (5), 1075–1085.

116 Ferreira-Pereira, A., Marco, S., Decottignies, A., Nader, J., Goffeau, A., and Rigaud, J.L. (2003) Three-dimensional reconstruction of the *Saccharomyces cerevisiae* multidrug

resistance protein Pdr5p. *The Journal of Biological Chemistry*, **278** (14), 11995–11999.

117 Kamis, A.B. (2005) Purification and electron microscopic studies of two ABC transporter proteins of clinical relevance. PhD thesis. University of Manchester, UK.

118 Mikhailov, M.V., Campbell, J.D., de Wet, H., Shimomura, K., Zadek, B., Collins, R.F., Sansom, M.S., Ford, R.C., and Ashcroft, F.M. (2005) 3-D structural and functional characterization of the purified KATP channel complex Kir6.2-SUR1. *The EMBO Journal*, **24**, 4166–4175.

119 Hofacker, M., Gompf, S., Zutz, A., Presenti, C., Haase, W., van der Does, C., Model, K., and Tampe, R. (2007) Structural and functional fingerprint of the mitochondrial ATP-binding cassette transporter Mdl1 from *Saccharomyces cerevisiae*. *The Journal of Biological Chemistry*, **282**, 3951–3961.

120 Higgins, C.F. (2007) Multiple molecular mechanisms for multidrug resistance transporters. *Nature*, **446**, 749–757.

121 Lage, H. (2003) ABC-transporters: implications on drug resistance from microorganisms to human cancers. *International Journal of Antimicrobial Agents*, **22** (3), 188–199.

122 Borst, P., Balzarini, J., Ono, N., Reid, G., de Vries, H., Wielinga, P., Wijnholds, J., and Zelcer, N. (2004) The potential impact of drug transporters on nucleoside-analog-based antiviral chemotherapy. *Antiviral Research*, **62** (1), 1–7.

123 Biedler, J.L. and Riehm, H. (1970) Cellular resistance to actinomycin D in Chinese hamster cells *in vitro*: cross-resistance, radioautographic, and cytogenetic studies. *Cancer Research*, **30** (4), 1174–1184.

124 Gottesman, M.M., Fojo, T., and Bates, S.E. (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. *Nature Reviews. Cancer*, **2** (1), 48–58.

125 Van Bambeke, F., Balzi, E., and Tulkens, P.M. (2000) Antibiotic efflux pumps. *Biochemical Pharmacology*, **60** (4), 457–470.

126 Paulsen, I.T., Brown, M.H., and Skurray, R.A. (1996) Proton-dependent multidrug efflux systems. *Microbiological Reviews*, **60** (4), 575–608.

127 van Veen, H.W. and Konings, W.N. (1998) The ABC family of multidrug transporters in microorganisms. *Biochimica et Biophysica Acta*, **1365** (1–2), 31–36.

128 Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J.P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J.C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R.H., Wilson, R.K., Hillier, L.W., McPherson, J.D., Marra, M.A., Mardis, E.R., Fulton, L.A., Chinwalla, A.T., Pepin, K.H., Gish, W.R., Chissoe, S.L., Wendl, M.C., Delehaunty, K.D., Miner, T.L., Delehaunty, A., Kramer, J.B., Cook, L.L., Fulton, R.S., Johnson, D.L., Minx, P.J., Clifton, S.W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J.F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., Gibbs, R.A., Muzny, D.M., Scherer, S.E., Bouck, J.B., Sodergren, E.J., Worley, K.C., Rives, C.M., Gorrell, J.H., Metzker, M.L., Naylor, S.L., Kucherlapati, R.S., Nelson, D.L., Weinstock, G.M., Sakaki, Y., Fujiyama, A., Hattori, M., Yada, T., Toyoda, A., Itoh, T., Kawagoe, C., Watanabe, H., Totoki, Y.,

Taylor, T., Weissenbach, J., Heilig, R., Saurin, W., Artiguenave, F., Brottier, P., Bruls, T., Pelletier, E., Robert, C., Wincker, P., Smith, D.R., Doucette-Stamm, L., Rubenfield, M., Weinstock, K., Lee, H.M., Dubois, J., Rosenthal, A., Platzer, M., Nyakatura, G., Taudien, S., Rump, A., Yang, H., Yu, J., Wang, J., Huang, G., Gu, J., Hood, L., Rowen, L., Madan, A., Qin, S., Davis, R.W., Federspiel, N.A., Abola, A.P., Proctor, M.J., Myers, R.M., Schmutz, J., Dickson, M., Grimwood, J., Cox, D.R., Olson, M.V., Kaul, R., Shimizu, N., Kawasaki, K., Minoshima, S., Evans, G.A., Athanasiou, M., Schultz, R., Roe, B.A., Chen, F., Pan, H., Ramser, J., Lehrach, H., Reinhardt, R., McCombie, W.R., de la Bastide, M., Dedhia, N., Blocker, H., Hornischer, K., Nordsiek, G., Agarwala, R., Aravind, L., Bailey, J.A., Bateman, A., Batzoglou, S., Birney, E., Bork, P., Brown, D.G., Burge, C.B., Cerutti, L., Chen, H.C., Church, D., Clamp, M., Copley, R.R., Doerks, T., Eddy, S.R., Eichler, E.E., Furey, T.S., Galagan, J., Gilbert, J.G., Harmon, C., Hayashizaki, Y., Haussler, D., Hermjakob, H., Hokamp, K., Jang, W., Johnson, L.S., Jones, T.A., Kasif, S., Kaspryzk, A., Kennedy, S., Kent, W.J., Kitts, P., Koonin, E.V., Korf, I., Kulp, D., Lancet, D., Lowe, T.M., McLysaght, A., Mikkelsen, T., Moran, J.V., Mulder, N., Pollara, V.J., Ponting, C.P., Schuler, G., Schultz, J., Slater, G., Smit, A.F., Stupka, E., Szustakowski, J., Thierry-Mieg, D., Thierry-Mieg, J., Wagner, L., Wallis, J., Wheeler, R., Williams, A., Wolf, Y.I., Wolfe, K.H., Yang, S.P., Yeh, R.F., Collins, F., Guyer, M.S., Peterson, J., Felsenfeld, A., Wetterstrand, K.A., Patrinos, A., Morgan, M.J., Szustakowki, J., de Jong, P., Catanese, J.J., Osoegawa, K., Shizuya, H., Choi, S., and Chen, Y.J., (2001) Initial sequencing and analysis of the human genome. *Nature*, **409** (6822), 860–921.

129 Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O., Yandell, M., Evans, C.A., Holt, R.A., Gocayne, J.D., Amanatides, P., Ballew, R.M., Huson, D.H., Wortman, J.R., Zhang, Q., Kodira, C.D., Zheng, X.H., Chen, L., Skupski, M., Subramanian, G., Thomas, P.D., Zhang, J., Gabor Miklos, G.L., Nelson, C., Broder, S., Clark, A.G., Nadeau, J., McKusick, V.A., Zinder, N., Levine, A.J., Roberts, R.J., Simon, M., Slayman, C., Hunkapiller, M., Bolanos, R., Delcher, A., Dew, I., Fasulo, D., Flanigan, M., Florea, L., Halpern, A., Hannenhalli, S., Kravitz, S., Levy, S., Mobarry, C., Reinert, K., Remington, K., Abu-Threideh, J., Beasley, E., Biddick, K., Bonazzi, V., Brandon, R., Cargill, M., Chandramouliswaran, I., Charlab, R., Chaturvedi, K., Deng, Z., Di Francesco, V., Dunn, P., Eilbeck, K., Evangelista, C., Gabrielian, A.E., Gan, W., Ge, W., Gong, F., Gu, Z., Guan, P., Heiman, T.J., Higgins, M.E., Ji, R.R., Ke, Z., Ketchum, K.A., Lai, Z., Lei, Y., Li, Z., Li, J., Liang, Y., Lin, X., Lu, F., Merkulov, G.V., Milshina, N., Moore, H.M., Naik, A.K., Narayan, V.A., Neelam, B., Nusskern, D., Rusch, D.B., Salzberg, S., Shao, W., Shue, B., Sun, J., Wang, Z., Wang, A., Wang, X., Wang, J., Wei, M., Wides, R., Xiao, C., Yan, C., Yao, A., Ye, J., Zhan, M., Zhang, W., Zhang, H., Zhao, Q., Zheng, L., Zhong, F., Zhong, W., Zhu, S., Zhao, S., Gilbert, D., Baumhueter, S., Spier, G., Carter, C., Cravchik, A., Woodage, T., Ali, F., An, H., Awe, A., Baldwin, D., Baden, H., Barnstead, M., Barrow, I., Beeson, K., Busam, D., Carver, A., Center, A., Cheng, M.L., Curry, L., Danaher, S., Davenport, L., Desilets, R., Dietz, S., Dodson, K., Doup, L., Ferriera, S., Garg, N., Gluecksmann, A., Hart, B., Haynes, J., Haynes, C., Heiner, C., Hladun, S., Hostin, D., Houck, J., Howland, T., Ibegwam, C., Johnson, J., Kalush, F., Kline, L., Koduru, S., Love, A., Mann, F., May, D., McCawley, S., McIntosh, T., McMullen, I., Moy, M., Moy, L., Murphy, B., Nelson, K., Pfannkoch, C., Pratts, E., Puri, V., Qureshi, H.,

Reardon, M., Rodriguez, R., Rogers, Y.H., Romblad, D., Ruhfel, B., Scott, R., Sitter, C., Smallwood, M., Stewart, E., Strong, R., Suh, E., Thomas, R., Tint, N.N., Tse, S., Vech, C., Wang, G., Wetter, J., Williams, S., Williams, M., Windsor, S., Winn-Deen, E., Wolfe, K., Zaveri, J., Zaveri, K., Abril, J.F., Guigo, R., Campbell, M.J., Sjolander, K.V., Karlak, B., Kejariwal, A., Mi, H., Lazareva, B., Hatton, T., Narechania, A., Diemer, K., Muruganujan, A., Guo, N., Sato, S., Bafna, V., Istrail, S., Lippert, R., Schwartz, R., Walenz, B., Yooseph, S., Allen, D., Basu, A., Baxendale, J., Blick, L., Caminha, M., Carnes-Stine, J., Caulk, P., Chiang, Y.H., Coyne, M., Dahlke, C., Mays, A., Dombroski, M., Donnelly, M., Ely, D., Esparham, S., Fosler, C., Gire, H., Glanowski, S., Glasser, K., Glodek, A., Gorokhov, M., Graham, K., Gropman, B., Harris, M., Heil, J., Henderson, S., Hoover, J., Jennings, D., Jordan, C., Jordan, J., Kasha, J., Kagan, L., Kraft, C., Levitsky, A., Lewis, M., Liu, X., Lopez, J., Ma, D., Majoros, W., McDaniel, J., Murphy, S., Newman, M., Nguyen, T., Nguyen, N., Nodell, M., Pan, S., Peck, J., Peterson, M., Rowe, W., Sanders, R., Scott, J., Simpson, M., Smith, T., Sprague, A., Stockwell, T., Turner, R., Venter, E., Wang, M., Wen, M., Wu, D., Wu, M., Xia, A., Zandieh, A., and Zhu, X., (2001) The sequence of the human genome. *Science*, **291** (5507), 1304–1351.

130 van Tellingen, O. (2001) The importance of drug-transporting P-glycoproteins in toxicology. *Toxicology Letters*, **120** (1–3), 31–41.

131 Lin, J.H. and Yamazaki, M. (2003) Clinical relevance of P-glycoprotein in drug therapy. *Drug Metabolism Reviews*, **35** (4), 417–454.

132 Kessel, D. and Bosmann, H.B. (1970) Effects of L-asparaginase on protein and glycoprotein synthesis. *FEBS Letters*, **10** (2), 85–88.

133 Juliano, R.L. and Ling, V. (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et Biophysica Acta*, **455** (1), 152–162.

134 Roninson, I.B., Abelson, H.T., Housman, D.E., Howell, N., and Varshavsky, A. (1984) Amplification of specific DNA sequences correlates with multi-drug resistance in Chinese hamster cells. *Nature*, **309**, 626–628.

135 Shen, D.W., Fojo, A., Chin, J.E., Roninson, I.B., Richert, N., Pastan, I., and Gottesman, M.M. (1986) Human multidrug-resistant cell lines: increased mdr1 expression can precede gene amplification. *Science*, **232** (4750), 643–645.

136 Gros, P., Ben Neriah, Y.B., Croop, J.M., and Housman, D.E. (1986) Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature*, **323** (6090), 728–731.

137 Gros, P., Croop, J., Roninson, I., Varshavsky, A., and Housman, D.E. (1986) Isolation and characterization of DNA sequences amplified in multidrug-resistant hamster cells. *Proceedings of the National Academy of Sciences of the United States of America*, **83** (2), 337–341.

138 Gros, P., Raymond, M., Bell, J., and Housman, D. (1988) Cloning and characterization of a second member of the mouse mdr gene family. *Molecular and Cellular Biology*, **8** (7), 2770–2778.

139 Ueda, K., Clark, D.P., Chen, C.J., Roninson, I.B., Gottesman, M.M., and Pastan, I. (1987) The human multidrug resistance (mdr1) gene. cDNA cloning and transcription initiation. *The Journal of Biological Chemistry*, **262** (2), 505–508.

140 Shen, D.W., Fojo, A., Roninson, I.B., Chin, J.E., Soffir, R., Pastan, I., and Gottesman, M.M. (1986) Multidrug resistance of DNA-mediated transformants is linked to transfer of the human mdr1 gene. *Molecular and Cellular Biology*, **6** (11), 4039–4045.

141 Cai, J. and Gros, P. (2003) Overexpression, purification, and functional characterization of ATP-binding cassette transporters in the yeast, *Pichia pastoris*.

Biochimica et Biophysica Acta, **1610** (1), 63–76.

142 Inaba, M. and Johnson, R.K. (1977) Decreased retention of actinomycin D as the basis for cross-resistance in anthracycline-resistant sublines of P388 leukemia. *Cancer Research*, **37** (12), 4629–4634.

143 Beck, W.T. (1987) The cell biology of multiple drug resistance. *Biochemical Pharmacology*, **36** (18), 2879–2887.

144 Beck, W.T. (1990) Multidrug resistance and its circumvention. *European Journal of Cancer*, **26** (4), 513–515.

145 Horio, M., Gottesman, M.M., and Pastan, I. (1988) ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. *Proceedings of the National Academy of Sciences of the United States of America*, **85** (10), 3580–3584.

146 Shapiro, A.B. and Ling, V. (1995) Reconstitution of drug transport by purified P-glycoprotein. *The Journal of Biological Chemistry*, **270** (27), 16167–16175.

147 Inaba, M., Kobayashi, H., Sakurai, Y., and Johnson, R.K. (1979) Active efflux of daunorubicin and adriamycin in sensitive and resistant sublines of P388 leukemia. *Cancer Res*, **39**, 2200–2203.

148 Schinkel, A.H. and Borst, P. (1991) Multidrug resistance mediated by P-glycoproteins. *Semin Cancer Biol*, **2**, 213–226.

149 van Zuylen, L., Verweij, J., Nooter, K., Brouwer, E., Stoter, G., and Sparreboom, A. (2000) Role of intestinal P-glycoprotein in the plasma and fecal disposition of docetaxel in humans. *Clinical Cancer Research*, **6** (7), 2598–2603.

150 Efferth, T. and Osieka, R. (1993) Clinical relevance of the MDR1 gene and its gene product, P-glycoprotein, for cancer chemotherapy: a meta-analysis. *Tumor Diagnostik und Therapie*, **14**, 238–243.

151 Jang, S.H., Wientjes, M.G., and Au, J.L. (2003) Interdependent effect of P-glycoprotein-mediated drug efflux and intracellular drug binding on intracellular paclitaxel pharmacokinetics: application of computational modelling. *The Journal of Pharmacology and Experimental Therapeutics*, **304** (2), 773–780.

152 Hendrikse, N.H., de Vries, E.G., Eriks-Fluks, L., van der Graaf, W.T., Hospers, G.A., Willemsen, A.T., Vaalburg, W., and Franssen, E.J. (1999) A new *in vivo* method to study P-glycoprotein transport in tumors and the blood–brain barrier. *Cancer Research*, **59** (10), 2411–2416.

153 Martin, C., Walker, J., Rothnie, A., and Callaghan, R. (2003) The expression of P-glycoprotein does influence the distribution of novel fluorescent compounds in solid tumour models. *British Journal of Cancer*, **89** (8), 1581–1589.

154 Cole, S.P., Bhardwaj, G., Gerlach, J.H., Mackie, J.E., Grant, C.E., Almquist, K.C., Stewart, A.J., Kurz, E.U., Duncan, A.M., and Deeley, R.G. (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer line. *Science*, **258**, 1650–1654.

155 Jedlitschky, G., Leier, I., Buchholz, U., Center, M., and Keppler, D. (1996) Transport of glutathione glucuronate and sulfate conjugates by the MRP gene-encoded conjugate export pump. *Cancer Research*, **56**, 988–994.

156 Jedlitschky, G., Leier, I., Buchholz, U., Center, M., and Keppler, D. (1994) ATP-dependent transport of glutathione S-conjugates by multidrug resistance-associated protein. *Cancer Research*, **54**, 4833–4836.

157 Westlake, C.J., Payen, L., Gao, M., Cole, S.P., and Deeley, R.G. (2004) Identification and characterization of functionally important elements in the multidrug resistance protein 1 COOH-terminal region. *The Journal of Biological Chemistry*, **279** (51), 53571–53583.

158 Bates, S.E. (2002) Solving the problem of multidrug resistance: ABC transporters in clinical oncology, in *ABC Proteins: From Bacteria to Man* (eds K. Kuchler, S.P. Cole,

and B. Holland), Academic Press, New York, pp. 65–80.

159 Wijnholds, J., de Lange, E.C.M., Scheffer, G.L., van der Berg, D.J., Mol, C.A.A.M., van der Valk, M., Schinkel, A.H., Scheper, R.J., Breimer, D.D., and Borst, P. (2000) Multidrug resistance protein 1 protects the choroid plexus epithelium and contributes to the blood–cerebrospinal fluid barrier. *The Journal of Clinical Investigation*, **105**, 279–285.

160 Schinkel, A.H. (1997) The physiological function of drug-transporting P-glycoproteins. *Seminars in Cancer Biology*, **8** (3), 161–170.

161 Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I., and Willingham, M.C. (1987) Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proceedings of the National Academy of Sciences of the United States of America*, **84** (21), 7735–7738.

162 Cordon-Cardo, C., O'Brien, J.P., Boccia, J., Casals, D., Bertino, J.R., and Melamed, M.R. (1990) Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. *The Journal of Histochemistry and Cytochemistry*, **38** (9), 1277–1287.

163 Regina, A., Koman, A., Piciotti, M., El Hafny, B., Center, M.S., Bergmann, R., Couraud, P.O., and Roux, F. (1998) Mrp1 multidrug resistance-associated protein and P-glycoprotein expression in rat brain microvessel endothelial cells. *Journal of Neurochemistry*, **71** (2), 705–715.

164 Sugawara, I., Koji, T., Ueda, K., Pastan, I., Gottesman, M.M., Nakane, P.K., and Mori, S. (1990) In situ localization of the human multidrug-resistance gene mRNA using thymine-thymine dimerized single-stranded cDNA. *Jpn J Cancer Res*, **81**, 949–955.

165 Sugawara, I., Kataoka, I., Morishita, Y., Hamada, H., Tsuruo, T., Itoyama, S., and Mori, S. (1988) Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as revealed by a monoclonal antibody, MRK 16. *Cancer Research*, **48** (7), 1926–1929.

166 Croop, J.M., Raymond, M., Haber, D., Devault, A., Arceci, R.J., Gros, P., and Housman, D.E. (1989) The three mouse multidrug resistance (mdr) genes are expressed in a tissue-specific manner in normal mouse tissues. *Molecular and Cellular Biology*, **9** (3), 1346–1350.

167 Borst, P. and Elferink, R.O. (2002) Mammalian ABC transporters in health and disease. *Annual Review of Biochemistry*, **71**, 537–592.

168 Chaudhary, P.M. and Roninson, I.B. (1991) Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. *Cell*, **66** (1), 85–94.

169 Wang, G., Pincheira, R., Zhang, M., and Zhang, J.T. (1997) Conformational changes of P-glycoprotein by nucleotide binding. *The Biochemical Journal*, **328** (Pt 3), 897–904.

170 Julien, M. and Gros, P. (2000) Nucleotide-induced conformational changes in P-glycoprotein and in nucleotide binding site mutants monitored by trypsin sensitivity. *Biochemistry*, **39** (15), 4559–4568.

171 Sonveaux, N., Vigano, C., Shapiro, A.B., Ling, V., and Ruysschaert, J.M. (1999) Ligand-mediated tertiary structure changes of reconstituted P-glycoprotein. A tryptophan fluorescence quenching analysis. *The Journal of Biological Chemistry*, **274** (25), 17649–17654.

172 Liu, R., Siemiarczuk, A., and Sharom, F.J. (2000) Intrinsic fluorescence of the P-glycoprotein multidrug transporter: sensitivity of tryptophan residues to binding of drugs and nucleotides. *Biochemistry*, **39** (48), 14927–14938.

173 Sonveaux, N., Shapiro, A.B., Goormaghtigh, E., Ling, V., and Ruysschaert, J.M. (1996) Secondary and tertiary structure changes of reconstituted P-glycoprotein. A Fourier transform

attenuated total reflection infrared spectroscopy analysis. *The Journal of Biological Chemistry*, **271** (40), 24617–24624.

174 Druley, T.E., Stein, W.D., and Roninson, I.B. (2001) Analysis of MDR1 P-glycoprotein conformational changes in permeabilized cells using differential immunoreactivity. *Biochemistry*, **40** (14), 4312–4322.

175 Druley, T.E., Stein, W.D., Ruth, A., and Roninson, I.B. (2001) P-glycoprotein-mediated colchicine resistance in different cell lines correlates with the effects of colchicine on P-glycoprotein conformation. *Biochemistry*, **40** (14), 4323–4331.

176 Ruth, A., Stein, W.D., Rose, E., and Roninson, I.B. (2001) Coordinate changes in drug resistance and drug-induced conformational transitions in altered-function mutants of the multidrug transporter P-glycoprotein. *Biochemistry*, **40** (14), 4332–4339.

177 Dawson, R.J. and Locher, K.P. (2007) Structure of the multidrug ABC transporter Sav1866 from *Staphylococcus aureus* in complex with AMP-PNP. *FEBS Letters*, **581**, 935–938.

178 Higgins, C.F. and Gottesman, M.M. (1992) Is the multidrug transporter a flippase? *Trends in Biochemical Sciences*, **17** (1), 18–21.

179 Homolya, L., Hollo, Z., Germann, U.A., Pastan, I., Gottesman, M.M., and Sarkadi, B. (1993) Fluorescent cellular indicators are extruded by the multidrug resistance protein. *The Journal of Biological Chemistry*, **268** (29), 21493–21496.

180 Raviv, Y., Pollard, H.B., Bruggemann, E.P., Pastan, I., and Gottesman, M.M. (1990) Photosensitized labeling of a functional multidrug transporter in living drug-resistant tumor cells. *The Journal of Biological Chemistry*, **265** (7), 3975–3980.

181 Eytan, G.D., Regev, R., Oren, G., and Assaraf, Y.G. (1996) The role of passive transbilayer drug movement in multidrug resistance and its modulation. *The Journal of Biological Chemistry*, **271** (22), 12897–12902.

182 Eytan, G.D., Regev, R., Oren, G., Hurwitz, C.D., and Assaraf, Y.G. (1997) Efficiency of P-glycoprotein-mediated exclusion of rhodamine dyes from multidrug-resistant cells is determined by their passive transmembrane movement rate. *European Journal of Biochemistry*, **248** (1), 104–112.

183 Choi, K., Chen, C.-J., Kriegler, M., and Roninson, I.B. (1988) An altered pattern of cross-resistance in multidrug resistant human cells results from spontaneous mutations in the mdr1 (P-glycoprotein) gene. *Cell*, **53**, 519–529.

184 Dhir, R., Grizzuti, K., Kajiji, S., and Gros, P. (1993) Modulatory effects on substrate specificity of independent mutations at the serine 939/941 position in predicted transmembrane domain 11 of P-glycoprotein. *Biochemistry*, **32**, 9492–9499.

185 Zhang, X., Collins, K.I., and Greenberger, L.M. (1995) Functional evidence that transmembrane 12 and the loop between transmembrane 11 and 12 form part of the drug-binding domain in P-glycoprotein encoded by MDR1. *The Journal of Biological Chemistry*, **270** (10), 5441–5448.

186 Bruggemann, E.P., Currier, S.J., Gottesman, M.M., and Pastan, I. (1992) Characterization of the azidopine and vinblastine binding site of P-glycoprotein. *The Journal of Biological Chemistry*, **267** (29), 21020–21026.

187 Demeule, M., Jodoin, J., Gingras, D., and Beliveau, R. (2000) P-glycoprotein is localized in caveolae in resistant cells and in brain capillaries. *FEBS Letters*, **466** (2–3), 219–224.

188 Demmer, A., Thole, H., Kubesch, P., Brandt, T., Raida, M., Fislage, R., and Tummler, B. (1997) Localization of the iodomycin binding site in hamster P-glycoprotein. *The Journal of Biological Chemistry*, **272** (33), 20913–20919.

189 Greenberger, L.M. (1993) Major photoaffinity labeling sites for iodoaryl azidoprazosin in P-glycoprotein are within or immediately C-terminal to transmembrane domains 6 and 12. *The Journal of Biological Chemistry*, **268**, 11417–11425.

190 Glossmann, H., Ferry, D.R., Striessnig, J., Goll, A., and Moosburger, K. (1987) Resolving the structure of the Ca^{2+} channel by photoaffinity labelling. *Trends in Pharmacological Sciences*, **8**, 95–100.

191 Tamai, I., and Safa, A.R. (1991) Azidopine noncompetitively interacts with vinblastine and cyclosporin A binding to P-glycoprotein in multidrug resistant cells. *J Biol Chem*, **266**, 16796–16800.

192 Martin, C., Berridge, G., Higgins, C.F., and Callaghan, R. (1997) The multi-drug resistance reversal agent SR33557 and modulation of vinca alkaloid binding to P-glycoprotein by an allosteric interaction. *British Journal of Pharmacology*, **122**, 765–771.

193 Ferry, D.R., Russell, M.A., and Cullen, M.H. (1992) P-glycoprotein possesses a 1,4-dihydropyridine-selective drug acceptor site which is allosterically coupled to a vinca-alkaloid-selective binding site. *Biochem Biophys Res Commun*, **188**, 440–445.

194 Malkhandi, J., Ferry, D.R., Boer, R., Gekeler, V., Ise, W., and Kerr, D.J. (1994) Dexniguldipine-HCl is a potent allosteric inhibitor of [3H]vinblastine binding to P-glycoprotein of CCRF ADR 5000 cells. *Eur J Pharmacol*, **288**, 105–114.

195 Loo, T.W., Bartlett, M.C., and Clarke, D.M. (2006) Transmembrane segment 1 of human P-glycoprotein contributes to the drug-binding pocket. *The Biochemical Journal*, **396**, 537–545.

196 Loo, T.W. and Clarke, D.M. (2000) Identification of residues within the drug-binding domain of the human multidrug resistance P-glycoprotein by cysteine-scanning mutagenesis and reaction with dibromobimane. *The Journal of Biological Chemistry*, **275**, 39272–39278.

197 Pleban, K., Kopp, S., Csaszar, E., Peer, M., Hrebicek, T., Rizzi, A., Ecker, G.F., and Chiba, P. (2005) P-glycoprotein substrate binding domains are located at the transmembrane domain/transmembrane domain interfaces: a combined photoaffinity labeling-protein homology modeling approach. *Molecular Pharmacology*, **67**, 365–374.

198 Loo, T.W., Bartlett, M.C., and Clarke, D.M. (2003) Permanent activation of the human P-glycoprotein by covalent modification of a residue in the drug-binding site. *The Journal of Biological Chemistry*, **278**, 20449–20452.

199 Loo, T.W., Bartlett, M.C., and Clarke, D.M. (2004) The drug-binding pocket of the human multidrug resistance P-glycoprotein is accessible to the aqueous medium. *Biochemistry*, **43**, 12081–12089.

200 Loo, T.W. and Clarke, D.M. (1997) Identification of residues in the drug-binding site of human P-glycoprotein using a thiol-reactive substrate. *The Journal of Biological Chemistry*, **272**, 31945–31948.

201 Rothnie, A., Storm, J., Campbell, J., Linton, K.J., Kerr, I.D., and Callaghan, R. (2004) The topography of transmembrane segment six is altered during the catalytic cycle of P-glycoprotein. *The Journal of Biological Chemistry*, **279**, 34913–34921.

202 Loo, T.W., Bartlett, M.C., and Clarke, D.M. (2006) Transmembrane segment 7 of human P-glycoprotein forms part of the drug-binding pocket. *The Biochemical Journal*, **399**, 351–359.

203 Loo, T.W. and Clarke, D.M. (1999) Identification of residues in the drug-binding domain of human P-glycoprotein. Analysis of transmembrane segment 11 by cysteine-scanning mutagenesis and inhibition by dibromobimane. *The Journal of Biological Chemistry*, **274**, 35388–35392.

204 Safa, A.R. (1993) Photoaffinity labeling of P-glycoprotein in multidrug-resistant cells. *Cancer Investigation*, **11**, 46–56.

205 Taguchi, Y., Kino, K., Morishima, M., Komano, T., Kane, S.E., and Ueda, K.

(1997) Alteration of substrate specificity by mutations at the His61 position in predicted transmembrane domain 1 of human MDR1/P-glycoprotein. *Biochemistry*, **36**, 8883–8889.

206 Taguchi, Y., Morishima, M., Komano, T., and Ueda, K. (1997) Amino acid substitutions in the first transmembrane domain (TM1) of P-glycoprotein that alter substrate specificity. *FEBS Letters*, **413**, 142–146.

207 Ma, J.F., Grant, G., and Melera, P.W. (1997) Mutations in the sixth transmembrane domain of P-glycoprotein that alter the pattern of cross-resistance also alter sensitivity to cyclosporin A reversal. *Molecular Pharmacology*, **51**, 922–930.

208 Song, J. and Melera, P.W. (2001) Transmembrane domain (TM) 9 represents a novel site in P-glycoprotein that affects drug resistance and cooperates with TM6 to mediate [^{125}I] iodoarylazidoprazosin labelling. *Molecular Pharmacology*, **60**, 254–261.

209 Hanna, M., Brault, M., Kwan, T., Kast, C., and Gros, P. (1996) Mutagenesis of transmembrane domain 11 of P-glycoprotein by alanine scanning. *Biochemistry*, **35**, 3625–3635.

210 Hafkemeyer, P., Dey, S., Ambudkar, S.V., Hrycyna, C.A., Pastan, I., and Gottesman, M.M. (1998) Contribution to substrate specificity and transport of nonconserved residues in transmembrane domain 12 of human P-glycoprotein. *Biochemistry*, **37**, 16400–16409.

211 Demeule, M., Laplante, A., Murphy, G.F., Wenger, R.M., and Beliveau, R. (1998) Identification of the cyclosporin-binding site in P-glycoprotein. *Biochemistry*, **37**, 18110–18118.

212 Loo, T.W. and Clarke, D.M. (1995) Identification of residues in the drug-binding site of human P-glycoprotein. *Journal of Biological Chemistry*, **272**, 31945–31948.

213 Taylor, A.M., Storm, J., Soceneantu, L., Linton, K.J., Gabriel, M., Martin, C., Woodhouse, J., Blott, E.J., Higgins, C.F., and Callaghan, R. (2001) Detailed characterization of cysteine-less P-glycoprotein reveals subtle pharmacological differences in function from wild-type protein. *British Journal of Pharmacology*, **134**, 1609–1618.

214 Rothnie, A., Storm, J., McMahon, R., Taylor, A., Kerr, I.D., and Callaghan, R. (2005) The coupling mechanism of P-glycoprotein involves residue L339 in the sixth membrane spanning segment. *FEBS Letters*, **579**, 3984–3990.

215 Brockman, R.W. (1963) Mechanisms of resistance to anticancer agents. *Advances in Cancer Research*, **57**, 129–234.

216 Hutchison, D.J. (1963) Cross resistance and collateral sensitivity studies in cancer chemotherapy. *Advances in Cancer Research*, **57**, 235–250.

217 Didziapetris, R., Japertas, P., Avdeef, A., and Petrauskas, A. (2003) Classification analysis of P-glycoprotein substrate specificity. *Journal of Drug Targeting*, **11**, 391.

218 Agrawal, M., Abraham, J., Balis, F.M., Edgerly, M., Stein, W.D., Bates, S., Fojo, T., and Chen, C.C. (2003) Increased 99mTc-sestamibi accumulation in normal liver and drug-resistant tumors after the administration of the glycoprotein inhibitor, XR9576. *Clinical Cancer Research*, **9** (2), 650–656.

219 Ganapathi, R. and Grabowski, D. (1983) Enhancement of sensitivity to adriamycin in resistant P388 leukemia by the calmodulin inhibitor trifluoperazine. *Cancer Research*, **43** (8), 3696–3699.

220 Goldberg, H., Ling, V., Wong, P.Y., and Skorecki, K. (1988) Reduced cyclosporin accumulation in multidrug-resistant cells. *Biochemical and Biophysical Research Communications*, **152** (2), 552–558.

221 Ramu, A., Fuks, Z., Gatt, S., and Glaubiger, D. (1984) Reversal of acquired resistance to doxorubicin in P388 murine leukemia cells by perhexiline maleate. *Cancer Research*, **44** (1), 144–148.

222 Tsuruo, T., Iida, H., Kitatani, Y., Yokota, K., Tsukagoshi, S., and Sakurai, Y. (1984) Effects of quinidine and related compounds on cytotoxicity and cellular accumulation of vincristine and adriamycin in drug-resistant tumor cells. *Cancer Research*, **44** (10), 4303–4307.

223 Tsuruo, T., Iida, H., Tsukagoshi, S., and Sakurai, J. (1981) Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Research*, **41**, 1967–1972.

224 Benson, A.B., 3rd, Trump, D.L., Koeller, J.M., Egorin, M.I., Olman, E.A., Witte, R.S., Davis, T.E., and Tormey, D.C. (1985) Phase I study of vinblastine and verapamil given by concurrent iv infusion. *Cancer Treatment Reports*, **69** (7–8), 795–799.

225 Cairo, M.S., Siegel, S., Anas, N., and Sender, L. (1989) Clinical trial of continuous infusion verapamil, bolus vinblastine, and continuous infusion VP-16 in drug-resistant pediatric tumors. *Cancer Research*, **49** (4), 1063–1066.

226 Bartlett, N.L., Lum, B.L., Fisher, G.A., Brophy, N.A., Ehsan, M.N., Halsey, J., and Sikic, B.I. (1994) Phase I trial of doxorubicin with cyclosporine as a modulator of multidrug resistance. *Journal of Clinical Oncology*, **12** (4), 835–842.

227 Verweij, J., Herweijer, H., Oosterom, R., van der Burg, M.E., Planting, A.S., Seynaeve, C., Stoter, G., and Nooter, K. (1991) A phase II study of epidoxorubicin in colorectal cancer and the use of cyclosporin-A in an attempt to reverse multidrug resistance. *British Journal of Cancer*, **64** (2), 361–364.

228 Plumb, J.A., Milroy, R., and Kaye, S.B. (1990) The activity of verapamil as a resistance modifier *in vitro* in drug resistant human tumour cell lines is not stereospecific. *Biochemical Pharmacology*, **39** (4), 787–792.

229 Bissett, D., Kerr, D.J., Cassidy, J., Meredith, P., Traugott, U., and Kaye, S.B. (1991) Phase I and pharmacokinetic study of D-verapamil and doxorubicin. *British Journal of Cancer*, **64** (6), 1168–1171.

230 Kornek, G., Raderer, M., Schenk, T., Pidlich, J., Schulz, F., Globits, S., Tetzner, C., and Scheithauer, W. (1995) Phase I/II trial of dexverapamil, epirubicin, and granulocyte-macrophage-colony stimulating factor in patients with advanced pancreatic adenocarcinoma. *Cancer*, **76** (8), 1356–1362.

231 Twentyman, P.R. and Bleehen, N.M. (1991) Resistance modification by PSC-833, a novel non-immunosuppressive cyclosporin [corrected]. *European Journal of Cancer*, **27** (12), 1639–1642.

232 Giaccone, G., Linn, S.C., Welink, J., Catimel, G., Stieltjes, H., van der Vijgh, W.J., Eeltink, C., Vermorken, J.B., and Pinedo, H.M. (1997) A dose-finding and pharmacokinetic study of reversal of multidrug resistance with SDZ PSC 833 in combination with doxorubicin in patients with solid tumors. *Clinical Cancer Research*, **3** (11), 2005–2015.

233 Boote, D.J., Dennis, I.F., Twentyman, P.R., Osborne, R.J., Laburte, C., Hensel, S., Smyth, J.F., Brampton, M.H., and Bleehen, N.M. (1996) Phase I study of etoposide with SDZ PSC 833 as a modulator of multidrug resistance in patients with cancer. *Journal of Clinical Oncology*, **14** (2), 610–618.

234 Friedenberg, W.R., Rue, M., Blood, E.A., Dalton, W.S., Shustik, C., Larson, R.A., Sonneveld, P., and Greipp, P.R. (2006) Phase III study of PSC-833 (valspodar) in combination with vincristine, doxorubicin, and dexamethasone (valspodar/VAD) versus VAD alone in patients with recurring or refractory multiple myeloma (E1A95): a trial of the Eastern Cooperative Oncology Group. *Cancer*, **106** (4), 830–838.

235 Hyafil, F., Vergely, C., Du Vignaud, P., and Grand-Perret, T. (1993) *In vitro* and *in vivo* reversal of multidrug resistance by GF120918, an acridonecarboxamide

derivative. *Cancer Research*, **53** (19), 4595–4602.

236 Roe, M., Folkes, A., Ashworth, P., Brumwell, J., Chima, L., Hunjan, S., Pretswell, I., Dangerfield, W., Ryder, H., and Charlton, P. (1999) Reversal of P-glycoprotein mediated multidrug resistance by novel anthranilamide derivatives. *Bioorganic & Medicinal Chemistry Letters*, **9** (4), 595–600.

237 Pusztai, L., Wagner, P., Ibrahim, N., Rivera, E., Theriault, R., Booser, D., Symmans, F.W., Wong, F., Blumenschein, G., Fleming, D.R., Rouzier, R., Boniface, G., and Hortobagyi, G.N. (2005) Phase II study of tariquidar, a selective P-glycoprotein inhibitor, in patients with chemotherapy-resistant, advanced breast carcinoma. *Cancer*, **104** (4), 682–691.

238 Pearce, H.L., Safa, A.R., Bach, N.J., Winter, M.A., Cirtain, M.C., and Beck, W.T. (1989) Essential features of the P-glycoprotein pharmacophore as defined by a series of reserpine analogs that modulate multidrug resistance. *Proceedings of the National Academy of Sciences of the United States of America*, **86** (13), 5128–5132.

239 Zamora, J.M., Pearce, H.L., and Beck, W.T. (1988) Physical–chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. *Molecular Pharmacology*, **33** (4), 454–462.

240 Scala, S., Akhmed, N., Rao, U.S., Paull, K., Lan, L.B., Dickstein, B., Lee, J.S., Elgemeie, G.H., Stein, W.D., and Bates, S.E. (1997) P-glycoprotein substrates and antagonists cluster into two distinct groups. *Molecular Pharmacology*, **51** (6), 1024–1033.

241 Seelig, A. (1998) A general pattern for substrate recognition by P-glycoprotein. *European Journal of Biochemistry*, **251** (1–2), 252–261.

242 Seelig, A. and Landwojtowicz, E. (2000) Structure–activity relationship of P-glycoprotein substrates and modifiers. *European Journal of Pharmaceutical Sciences*, **12**, 31–40.

243 Raad, I., Terreux, R., Richomme, P., Matera, E.L., Dumontet, C., Raynaud, J., and Guilet, D. (2006) Structure–activity relationship of natural and synthetic coumarins inhibiting the multidrug transporter P-glycoprotein. *Bioorganic and Medicinal Chemistry*, **14** (20), 6979–6987.

244 Kaiser, D., Smiesko, M., Kopp, S., Chiba, P., and Ecker, G.F. (2005) Interaction field based and hologram based QSAR analysis of propafenone-type modulators of multidrug resistance. *Medicinal Chemistry*, **1** (5), 431–444.

245 Labrie, P., Maddaford, S.P., Fortin, S., Rakhit, S., Kotra, L.P., and Gaudreault, R.C. (2006) A comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) of anthranilamide derivatives that are multidrug resistance modulators. *Journal of Medicinal Chemistry*, **49** (26), 7646–7660.

246 Cianchetta, G., Singleton, R.W., Zhang, M., Wildgoose, M., Giesing, D., Fravolini, A., Cruciani, G., and Vaz, R.J. (2005) A pharmacophore hypothesis for P-glycoprotein substrate recognition using GRIND-based 3D-QSAR. *Journal of Medicinal Chemistry*, **48** (8), 2927–2935.

247 Aller, S.G., Yu, J., Ward, A., Weng, Y., Chittaboina, S., Zhuo, R., Harrell, P.M., Trinh, Y.T., Zhang, Q., Urbatsch, I.L., and Chang, G. (2009) Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science*, **323**, 1718–1722.

248 Kadaba, N.S., Kaiser, J.T., Johnson, E., Lee, A., and Rees, D.C. (2008) The high-affinity E. coli methionine ABC transporter: structure and allosteric regulation. *Science*, **321**, 250–253.

249 Khare, D., Oldham, M.L., Orelle, C., Davidson, A.L., and Chen, J. (2009) Alternating access in maltose transporter mediated by rigid-body rotations. *Mol Cell*, **33**, 528–536.