rijksuniversiteit groningen

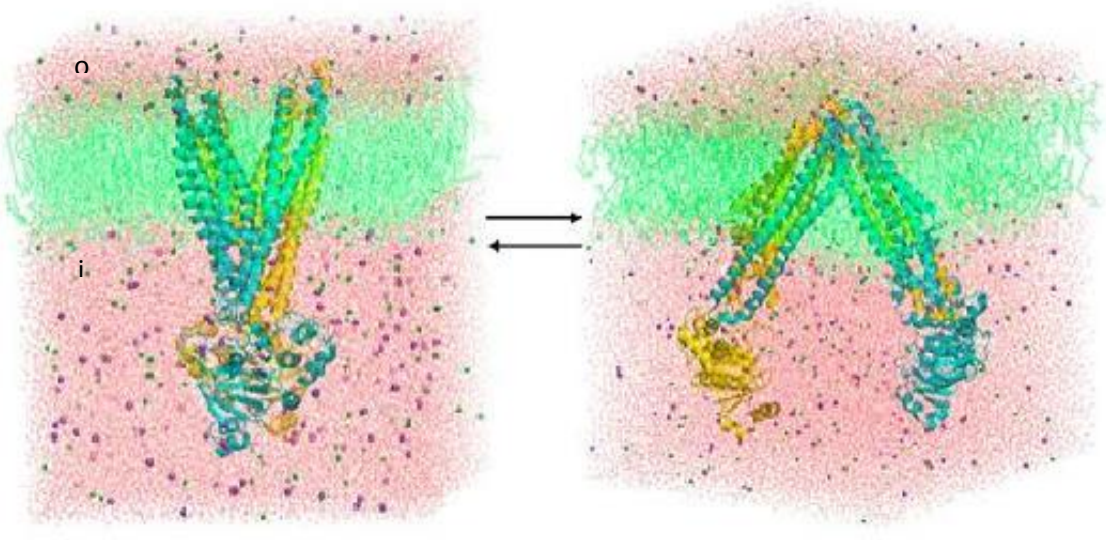
GBB

Master Essay July/August 2013

The Molecular Basis for Substrate Poly-Specificity of P-glycoprotein

M.S. Overgaauw

Supervisor: Prof. Dr. A.J.M. Driessen



Source image: Mchaourab et. al. (mpsdc)

TABLE OF CONTENTS

Abstract……2

INTRODUCTION……2-3

STRUCTURAL CHARACTERIZATION……3-9
- Crystal structures of the apo form of P-glycoprotein……4-5
- Inhibitor-based poly-specific drug-binding……5-7
- Drug transport from the lipid bilayer ……7
- Structure-function relationships of P-gp……7-9

ELUCIDATING THE DRUG EFFLUX MECHANISM……9-14
- ATPase reaction intermediates in the catalytic cycle of P-gp……10-12
- Interdomain cross-talk mediates drug transport…… 13-14

CHARACTERIZATION OF THE DRUG-BINDING SITES……14-19
- Refining the drug-entry portals of P-gp ……14-16
- P-gp efflux modulation and drug promiscuity ……16-19

SUMMARY……19

DISCUSSION AND PERSPECTIVES……19-20

REFERENCES

Abstract
P-glycoprotein is an ATP-binding cassette (ABC) transporter best known for its involvement in multidrug resistance (MDR) in numerous cancers as conferred by its ability to transport a wide variety of unrelated drugs out of the cell. The development of inhibitors and modulators as a defense against MDR relies strongly on the knowledge obtained from structural and functional studies. Despite previous experimental efforts, there is still a great deal of information missing concerning the protein's structure, mechanism of action, and features of drug-binding sites. This essay reviews several studies that contributed largely to unraveling these major facets. Crystal structures of P-glycoprotein from mouse and *C. elegans* confirmed the transporter arranges into two homologous halves that each consists of one transmembrane domain (TMD) and one nucleotide-binding domain (NBD). Studies including radioligand binding assays and disulfide cross-linking indicated that ATP-driven dimerization of both NBDs induces structural rearrangements that lowers the binding affinities of drug molecules, e.g. verapamil and vinblastine. Finally, a series of docking studies allowed the identification of binding features that distinguish transported substrates from modulators.

INTRODUCTION

Cellular efflux is the movement of solute substances in the direction of the extracellular space through their translocation across extra- and intracellular membranes. Among proteins that catalyze this type of transcellular transport, the ABC transporters are the most predominant and constitute the largest transmembrane protein family with representatives in all branches of life (reviews: Ref 1, 2). They directly couple energy from ATP hydrolysis to drive the translocation of their substrates across cellular membranes, sometimes against a steep concentration gradient. In eukaryotes, ABC transporters primarily serve as efflux pumps that excrete toxins and drugs. The first sequence sequence determination of an ABC transporter in eukaryotes was that of human P-glycoprotein (permeability glycoprotein, P-gp) and showed strong similarity to earlier studied bacterial transporters[3,4]. P-glycoprotein is the most studied efflux pump and over the past decades has risen major clinical relevance owing to its impressive and pivotal role in multidrug resistance relative to other transporters.

The human P-glycoprotein, also known as multidrug resistance protein 1 (MDR1) or ABC subfamily B member 1 (*ABCB1*), is the 170 kDa product of the *ABCB1* gene and is a member of the multidrug resistance/transporter of antigen presentation (MDR/TAP) family. They transport a vast array of structurally diverse compounds that range in size from ±330 up to 4000 daltons[5,6] and are commonly hydrophobic with cationic properties[7]. Xenobiotics form a majority of the substrate-spectrum, the most prominent of which include anticancer drugs, cardiac drugs, Ca^{2+} channel blockers, HIV protease inhibitors, and macrolide antibiotics[8,9]. Like each drug transporter, P-gp has a specific pattern of constitutive expression at a number of tissues. Earlier localization studies conducted in human tissues suggested a low expression of P-gp in most examined tissues[10]. However, the transporter was found to be highly concentrated on the apical (luminal) side of certain cells of secretory tissues including colon, small intestine, kidney and liver[10]. After drug entry in the blood circulation, P-gp limits drug absorption and distribution in these tissues by promoting drug clearance into urine, bile, and the intestinal lumen. As a result, tissues are protected from excessive penetration and subsequent accumulation of potentially toxic xenobiotics.

Other functional sites of P-gp that are of particular importance for drug disposition are blood-tissue barriers, e.g. the blood-brain barrier and blood-testis barrier (review: Ref. 11). Over 2 decades ago, in vivo studies clearly indicated a significant accumulation of xenobiotics in the brains of P-glycoprotein-deficient mice as opposed to mice with normal P-glycoprotein expression[12,13]. This marked the importance of P-glycoprotein-mediated drug efflux as a defensive mechanism at the blood-brain

barrier. However, because this particular defensive mechanism covers such a broad range of drugs and drug conjugates, drug efflux by P-gp has formed a limiting factor in drug bioavailability.

The substrate poly-specificity of P-gp is consistent with a multidrug resistance (MDR) phenotype in many human cancer cell lines. As mentioned, P-gp inhibits drug accumulation and, thereby, maintains intracellular drug concentrations below cytotoxic levels. As a consequence, many cancer cells acquire resistance against cytostatic agents that fall under the substrate poly-specificity of P-gp[14]. Cytostatic agents that are frequently subjected to MDR are the anthracyclines (doxorubicin, daunorubicin), Vinca alkaloids, epipodophyllotoxins (etoposide), and taxanes (paclitaxel). The most prominent and studied mechanism of MDR in cancer cells is P-gp overexpression. Numerous targets have been identified for therapeutic interventions of MDR, several of which inhibit factors that promote overexpression of P-gp[15,16]. Other drugs, e.g. macrolide antibiotics, directly reduce the efflux rate of P-gp substrates such as digoxin[17]. However, a rapid development of specific P-gp inhibitors/modulators with minimal adverse effects requires more knowledge on the structural basis underlying poly-specific drug-binding ascribed to this protein. Elucidating the structure-function relationships will also aid to characterize the drug-binding sites of P-gp and generate novel models describing drug efflux mechanisms. A wide variety of studies focusing on diverse functional aspects of drug-protein interactions and drug efflux has proven necessary in achieving these goals. X-ray structures of P-gp[18,19] formed a crucial framework in unraveling drug-protein interactions as investigated by molecular dynamics (MD) simulations[20] and docking studies[21]. In addition, MD simulations highlighted the importance of the lipid membrane and a linker sequence in the stability of P-gp[20]. This essay aims to review and discuss the knowledge obtained from P-glycoprotein research as above-described. These studies contributed to a better understanding of the poly-specific nature of P-glycoprotein.

STRUCTURAL CHARACTERIZATION

P-glycoprotein is expressed as a 170 kDa integral plasma membrane protein consisting of 1280 amino acids. During early topology studies, P-gp was predicted from its primary sequence to be comprised of two transmembrane domains (TMD) and two cytoplasmic nucleotide binding domains (NBD). Each of the TMDs consists of six membrane-spanning segments (putative α-helices) and are separated by hydrophilic loops[22,23]. Moreover, internal repeats of amino acids inside the TMD sequences predict a 2-fold pseudo symmetry in the P-gp structure. Both TMDs are thought to form the translocation pathway of solutes and to play a major role in determining substrate-specificity. The cytoplasmic NBDs contain the highly conserved motifs, including Walker A and B[24,25], and couple ATP binding and hydrolysis to conformational changes that enable the transport process[26].

The knowledge on most ABC transporters, like concisely presented above, is extracted from an appreciable body of biochemical and genetic data. However, a better understanding of the protein's function requires a more detailed picture of its structure. The first experimental insight into the three-dimensional structure of P-gp, and of any ATP binding cassette transporter, originated from projections of single-particles measured by electron microscopy[27,28]. This initial P-gp structure was determined at 2.5 nm resolution (25 Å) which was sufficient to derive a structural model that described the overall shape of the protein. Although this model was entirely consistent to the predicted structural features as derived from available biochemical and genetic data, it remotely approximates the P-gp structure at atomic level. Structure determinations at higher resolutions, as accomplished by X-ray crystallography, provides a closer approximation to a (near) atomic model.

Crystal structures of the apo form of P-glycoprotein

To date, crystal structures have been resolved for mouse[18] and *C. elegans* P-gp (latest published)[19] at 3.8 and 3.4 Å resolution, respectively. Mouse P-gp shows 87% sequence identity with human P-gp in a drug-binding-competent state, whereas *C. elegans* P-gp is 46% identical in a similar conformation. Both studies revealed similar apo P-gp structures in a pre-transport state. Nucleotide-free P-gp crystallizes as a single polypeptide into an inward-facing conformation of two structurally homologous "halves" with pseudo two-fold molecular symmetry (Figure 1). Each of these constitute halves, containing one TMD and one NBD, are predicted to have a spanning of ±136 Å perpendicular to and and ±70 Å in the plane of the membrane. An average distance of ±30 Å separates the two NBDs. In crystal form, two P-gp molecules were found in the asymmetric unit. As both structures were highly similar, only one of them is presented below (Figure 1).

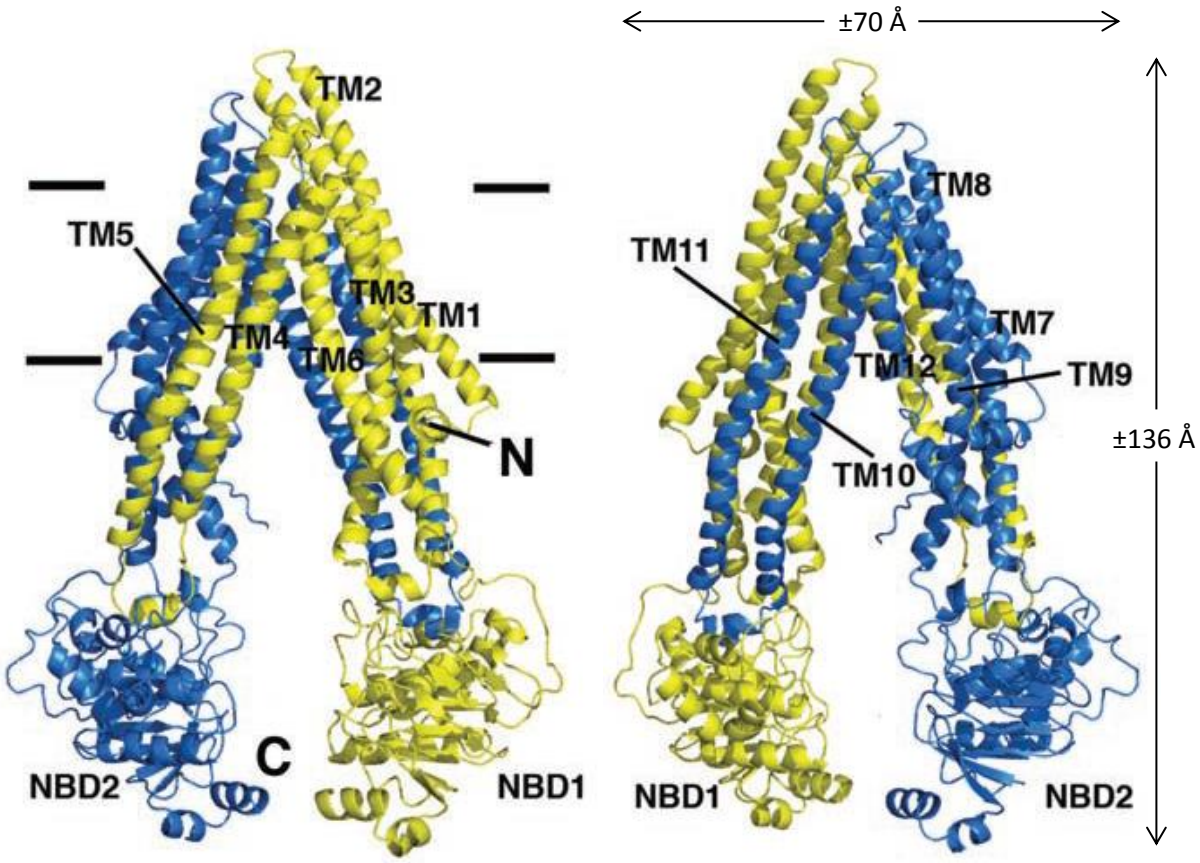


Figure 1: Mouse P-gp structure. Stereo views in ribbon presentation at the front (left) and back (right) of the molecule. Transmembrane helices TM1 to 12 are labeled accordingly. The N-terminal half is colored yellow and the C-terminal half is colored blue. The lipid bilayer is approximated by horizontal bars in the left figure.
Source: Aller et. al. 2009 (Ref. 20)

The inward-facing conformation gives rise to a large internal cavity that is open to the cytoplasm and continuous with the membrane inner leaflet. This intramembranar (lateral) region of the cavity occurs at both opposite sides of the protein. The overall architecture of the internal cavity allows for drug entry from the cytoplasm and directly from the membrane inner leaflet. For both structures, the

lining of the presumptive drug-binding pocket (DBP) is predominantly made up of hydrophobic and aromatic residues. This enables accommodation of mainly hydrophobic drugs as is consistent to most P-gp substrates partitioning in the cellular membrane. For mouse P-gp, a volume of ±6000 $Å^3$ was estimated for the internal cavity part running through the membrane inner leaflet. A pocket of this substantial volume has previously been shown to bind at least two different drugs simultaneously and, by suggestion, at different regions in the common DBP[29]. Stereo views of the drug-entry portals within the membrane inner leaflet are presented in figure S12 of reference 20.

Structural alignments of mouse P-gp and *C. elegans* P-gp structures revealed a substantial degree of overlap between the transmembrane helices TM1, 2, 6, 7, 8, and 11 (overlay figure not shown in this essay). The two structures showed different conformations in helices TM9, 10, and 12, making these helices incomparable. Additional helices that agreed well in structural alignments were TM3, 4, and 5. Important differences in these helices, however, were due to register shifts in model building. As a result, the orientations of TM3, 4, and 5 with respect to the drug-translocation pathway in both crystal structures are incompatible with one another. Although not reported in the research paper, these incompatibilities in helices TM3, 4, and 5 of both structures allegedly dot not translate into functional dissimilarities, e.g. different drug-binding affinities among mouse and *C. elegans* P-gp. In addition, the cytoplasmic linker region that connects both homologous halves of P-gp is missing in both structures, indicating it is possibly not backed up by electron density. However, such negative density would be indicated by anomalous Fourier peaks. An electron density map for the mouse P-gp structure is presented and lacks such a peak (Figure S7 in reference 18). Nonetheless, both studies do not mention the missing linker region. As shall be discussed later, MD simulations succeeded to complete the mouse P-gp model[20].

Inhibitor-based poly-specific drug-binding

Crystallization of mouse P-gp was primarily aimed at capturing a structural view representing the biochemical basis for poly-specificity[18]. As a strategy, Aller *et al.* cocrystallized mouse P-gp in complex with two stereoisomers of cyclic hexpeptide inhibitors, abbreviated QZ59-RRR and QZ59-SSS. As indicated by transport assays, both stereoisomer compounds inhibited verapamil-stimulated ATPase activity of P-gp in a concentration-dependent manner. Furthermore, P-gp overexpression in Chinese hamster ovary cells provided a rescue from growth inhibition by the anticancer drug colchicine. However, presence of both QZ59 compounds in the medium sensitized these cells to colchicine, reversing the MDR phenotype to differing degrees in a concentration-dependent fashion. Similar assays performed in *Spodoptera frugiperda* (Sf9) cells overexpressing *C. elegans* P-gp showed cellular resistance against human anticancer drugs such as actinomycin D and paclitaxel (Taxol)[19]. This MDR phenotype revealed a functional similarity between *C. elegans* and human P-gp despite their substrate specificity profiles only partly overlap.

Cocrystallization of P-gp molecules with the individual QZ59 compounds demonstrated distinct drug-binding sites in the internal cavity. Both cocrystal structures elucidated the relevance of the inward-facing conformation of P-gp for binding of the two QZ59 stereoisomers and, presumably, other drugs. Therefore, inhibition by both compounds proceeds competitively rather than allosterically. Importantly, P-gp is able to discriminate between both QZ59-RRR and QZ59-SSS stereoisomers which ultimately results into different binding sites, orientation, and stoichiometry (Figure 2). The binding of QZ59-RRR occurs only at the center of the P-gp molecule between helices TM6 and TM12, which previously have been identified in drug-binding interactions[30,31]. The QZ59-SSS stereoisomer, however, binds at higher and lower sites of the P-gp molecule. A portion of the ligand bound at the higher site was disordered in both structures in the asymmetric unit (not shown here).
The binding of QZ59-RRR at the middle site and QZ59-SSS at the higher site is mediated predominantly by hydrophobic residues. Only QZ59-SSS in the lower site is surrounded by three polar residues. The residues occur on a number of surrounding TM helices, most of which are shared by both ligands for their binding. Given these shared binding conditions along with the relatively low

resolution (3.8 Å) of the structure and the disordered portion of QZ59-SSS at the higher binding site, it is readily conceivable that binding of both QZ59 compounds is interchangeable for at least the middle (QZ59-RRR) and higher site. However, the authors do not postulate that another cocrystal structure may demonstrate swapped binding locations of the QZ59 compounds as such.

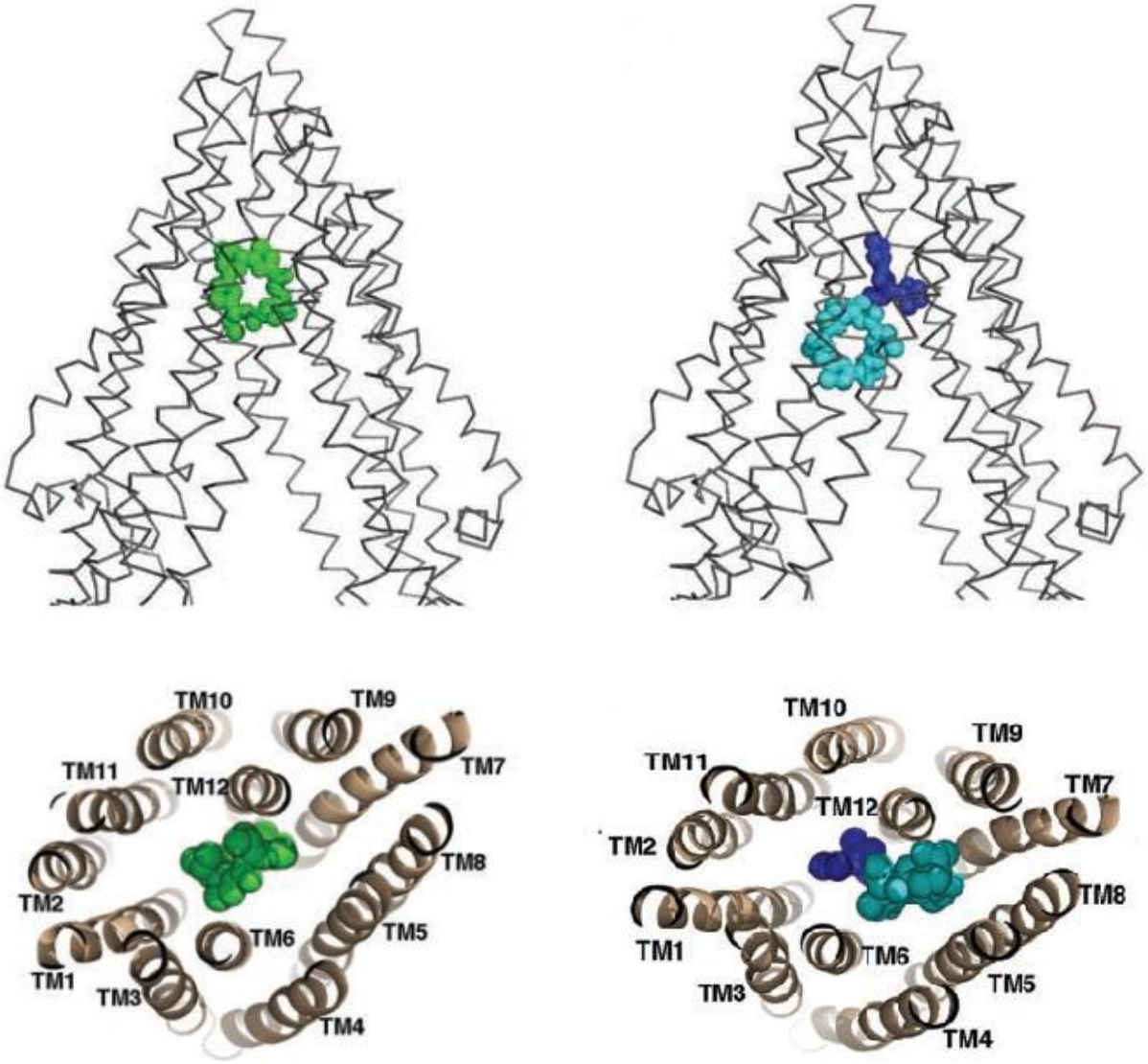


Figure 2: Binding locations for novel cyclic hexapeptide inhibitors in mouse P-gp. Top figures: binding location of QZ59-RRR (green spheres) and two binding locations of the QZ59-SSS molecules (blue and cyan spheres). Note, the QZ59-SSS molecule occupying the higher binding site in the internal cavity (blue spheres) is incomplete as this region was absent in both copies of P-gp in the asymmetric unit. Bottom figures: stereo views in ribbon presentation showing the interactions between QZ59 molecules and TM helices as seen from the intracellular face of the protein, looking into the drug-binding chamber. Note that both inhibitor molecules dock between helices TM6 and TM12, demonstrating their importance in drug-binding.

A flexibility in the hydrophobic residues on the transmembrane helices allowing random binding of the QZ59 compounds is consistent with a reduction in enantioselectivity. This is already demonstrated by the lowered restriction for the binding of QZ59-SSS, recognizing either the higher or

lower site within the drug-binding cavity. Moreover, the isopropyl groups of the QZ59-RRR/SSS molecules are pointed towards TM9 and TM12 (not shown here). Despite this apparent flexibility in strereoselectivity, the difference density maps show different specific functional roles for different residues governing protein-ligand interactions. For instance, the phenyl moiety of F332 contacts QZ59-RRR locating in the middle site and QZ59-SSS in the upper site. This flexibility in contacting both stereoisomers at different locations is accomplished by two side-chain rotamer conformations in F332. In addition, the small spacing between the higher and lower binding locations further facilitates both residue-ligand interactions. Though not mentioned in the article, the difference density map clearly demonstrates both contributing factors in contacting the two QZ59 stereoisomers. An important role in ligand binding is fulfilled by V978 (human V982) as it lies in close proximity to all three binding locations and, therefore, contacts all QZ59 compounds. Along with residue F724 (human F728) V978 is protected from methanethiosulfonate-verapamil (MTS-verapamil)labeling by verapamil, which underlines their importance in drug binding. It was suggested that the binding site for verapamil overlaps with all three binding sites of the QZ59 compounds.

While the upper half of the DBP is stated to contain primarily hydrophobic and aromatic residues, the lower half has polar and charged residues. The lower half of the chamber may allow binding of hydrophobic and positively charged substrates. Similar interactions were found in the poly-specific DBP of transporters QacR and EmrE where charged residues like glutamate were used to neutralize a variety of drugs[32,33]. Moreover, several cross studies on the DBP identified highly conserved residues in human and mouse P-gp that mediate verapamil-binding (see also figure S1 in reference 18)[34,35]. This indicates these residues are involved in a common mechanism for poly-specific drug recognition.

Drug transport from the lipid bilayer

As demonstrated from apo structures of both mouse and *C. elegans* P-gp, membrane-partitioning substrates could in principle gain access in the drug-entry portals crossing the membrane inner leaflet. A particular mechanism for this crucial step of the drug-transport cycle is called the 'hydrophobic vacuum' model[36]. This is currently the most accepted model for the efflux mechanism as most P-gp substrates are highly concentrated in the membrane, with partition coefficients ranging from 100 to 10,000[37]. The transport function of *C. elegans* P-gp was examined in context with its lipid environment[19]. Transport assays, like above-described, demonstrated a strong dependency of drug-stimulated ATPase activity on the lipid environment. ATPase activity showed a 4,000-fold increase in sensitivity for actinomycin D when P-gp was overexpressed in yeast cells compared to using detergent-purified P-gp. For paclitaxel, an increase in sensitivity of 100-fold was observed. The baseline activity of additional ATPases in the cellular membranes of yeast probably contributed to the lower degree of drug stimulation. Whether the activity peak in ATPase belonging to P-gp drastically exceeds the baseline activity of other ATPases is not assessed in this study. Nonetheless, these observations strongly supports the hypothesized drug-entry from the membrane. Importantly, the authors state that the transport of membrane-partitioning substrates is consistent with poly-specific drug-binding as is known for P-gp. The ability to transport a wide variety of compounds implies a low intrinsic substrate affinity which, at its turn, is consistent to a need for high local substrate concentrations as is the case for P-gp substrates embedded in the membrane.

Structure-function relationships of P-gp

A number of notable structural characteristics have been observed in the *C. elegans* P-gp structure, some of which are implied in several functions. All of these properties are representative in a number of other organisms. An interesting feature of *C. elegans* P-gp, one that was also found in mouse P-gp and the bacterial exporter MsbA[38] , is the block of one lateral opening by the insertion of an amino-terminal helical hairpin[19]. Although this obstruction restricts the access of membrane-partitioning drugs, truncation of the hairpin does not lead to an altered dependency on drug concentrations in

cytotoxic assays[19]. However, a reduced maximal level of stimulation ATPase activity was observed compared to full-length P-gp.

The second structural feature observed in *C. elegans* P-gp is a discontinuity within helices TM10 and TM12 that form the lining of the lateral opening. Two possible functions for these interrupted transmembrane helices were hypothesized. The first is that discontinuous secondary structures like these may potentiate a greater number of residue-substrate interactions in the translocation pathway. This postulate was based on a previous observations of discontinuous TM helices in other transporters that bound substrates[39]. A second possible reason is that these helices function as flexible hinges that gate the pathway and facilitate conformational changes resulting into drug transport, or both. Both possibilities could be tested by molecular dynamics (MD) simulations. As shall be discussed later, MD simulations were also used to identify conformational changes associated with drug transport[20].

The third structural feature recognized is the TMD-NBD interface which is the connection between the TMD and the NBD according a 'ball-and-socket' joint. The joint is formed by a cytoplasmic helix from the TMD that is known as the coupling helix or EAA loop (the ball). This helix docks into a cleft on the NBD surface (the socket). This typical connection has been detected in all ABC transporters for which structures have been resolved, most recently *C. elegans* P-gp. In this structure, and a number of other ABC exporters, intracellular helices IH1 to IH4 fulfill the function of the coupling helix and create an extensive interaction surface between the TMDs and NBDs. For both TMD-NBD1 and TMD-NBD2, the structure showed that three highly conserved residues are engaged in a network of salt bridge interactions (Figure 3b,c). Similar salt bridge interactions were found in crystal structures of the maltose importer and seem to be central to tethering a pivot point in conformational changes that were accompanied by rotations of the EAA loop (coupling helix) inside the cleft[40]. These salt bridge interactions were also shown to be crucial in maintaining the assembly of the transmembrane subunit (MalF) and the NBD (MalK). Elimination of the salt bridges by double mutations resulted into the dissociation of the MalK subunit[41]. A double mutation as such will point out a similar stabilizing function for P-gp.

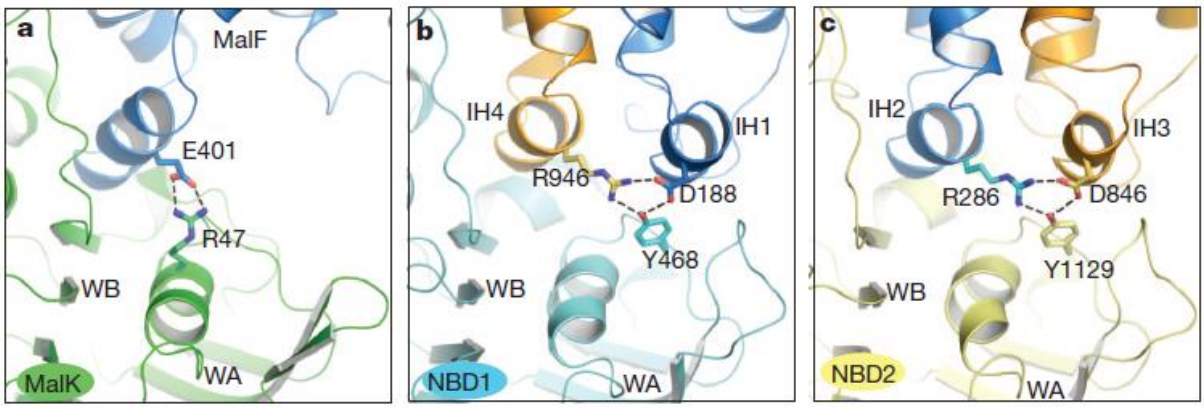


Figure 3: Interactions in the TMD-NBD interface. a) TMD-NBD interactions in the maltose importer are governed by Arg47 and the conserved glutamate residue Glu401 in the EAA loop. MalF and MalK designate the transmembrane and NBD subunits respectively. **b, c)** Interactions between TMD and NBD1 (b) and NBD2 (c) in *C. elegans* P-gp, showing the three highly conserved residues engaging in a network of salt bridges. Dashed lines indicate salt bridges and hydrogen bonds. WA) Walker A motif; WB) Walker B motif.

Finally, a homology model of human P-gp was created based on the determined *C. elegans* P-gp structure. The structure model located Phe335 to the apex of the DBP (that is, the top of the lateral opening). Van der Waals (vdw) interactions between Phe335 and three surrounding residues (Tyr310,

Phe314, and Ile218) stabilize the inward-facing conformation. Previous studies showed that Phe335Ala mutants resulted into higher ATPase activity but similar or lower drug resistance[42,43]. These effects would be due to the loss of vdw interactions in the apex of the translocation pathway, which destabilized the inward-facing conformation and thus triggered the conformational transition into the outward-facing conformation (closed to the cytoplasm). In parallel to this, biochemical and structural evidence suggested that the products of ATP hydrolysis, ADP and P_i, destabilize the dimerization of the NBDs[44] and, thus, has the same basic effect as the Phe335Ala mutant. This description of the Phe335Ala mutant fits to mutants previously identified in the maltose importer which allowed constitutive ATP hydrolysis in the absence of the binding protein[45].

Given the observation above-mentioned that Phe335Ala did not lead to a dramatic reduction in drug resistance, it is less likely that F335 in P-gp is majorly involved in the drug transport mechanism. Nonetheless, it is conceivable that a certain drug compound causes a similar break in vdw interactions upon binding specific residues which, at its turn, leads to conformational changes that allow substrate release. What type of protein-substrate interactions are known and how they are coupled to ATP binding and hydrolysis that power drug transport shall be discussed throughout the next chapter.

ELUCIDATING THE DRUG EFFLUX MECHANISM

As briefly touched in the previous section, the catalytic cycle of P-glycoprotein involves conformational changes in the TMDs whereby a high-affinity DBP in the cytoplasmic-facing conformation is converted into an extracellular-facing conformation bearing low affinity. These conformational changes are associated with ATP binding and hydrolysis occurring at both C- and N-terminal NBDs. Aller *et. al*. derived a model from the crystal structure of Sav1866 and MsbA

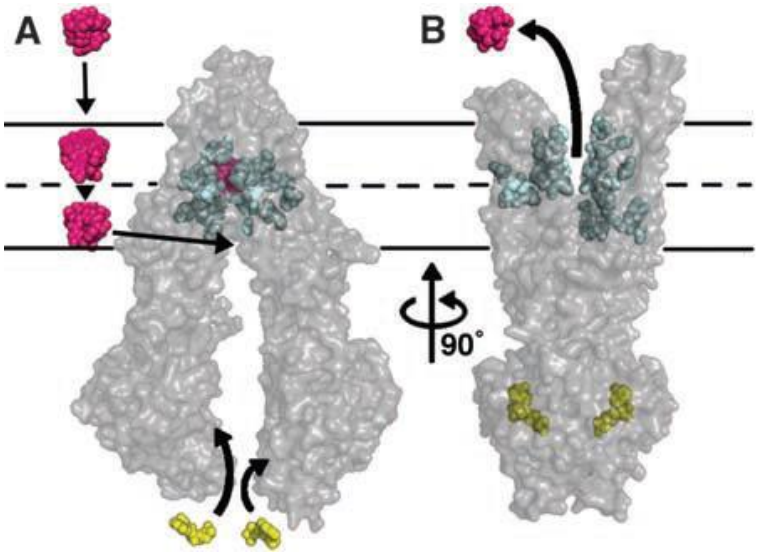


Figure 4: Model of the drug efflux mechanism for P-gp. A) Substrate (magenta) permeates the lipid bilayer and enters the accessible drug-entry portal. Residues in the drug-binding pocket (cyan) interact with either QZ59 stereoisomers and verapamil. **B)** Binding of ATP (yellow) induces a large conformational change, which dimerizes both NBDs and presents the substrate and resideus lining the drug-binding pocket to the extracellular space. Escape of the substrate from the portal back into the inner leaflet is sterically occluded.

depicting the outward-facing conformation (Figure 4B)[46,47]. This model gives a rough description of the catalytic cycle from substrate binding to release into the extracellular space. The binding of ATP, stimulated by substrate-binding, drives the dimerization of the NBDs by drawing them close

together. The conformational change that is associated with the dimerization of the NBDs is instantly transmitted to the TMDs, resulting in the outward-facing conformation of the protein. This exposes the bound substrate to the extracellular space in which it is finally released. Using a vast body of mutational and biochemical data, earlier transport models have been revisited and incorporated with new concepts that include dimerization of the NBDs upon ATP binding.

ATPase reaction intermediates in the catalytic cycle of P-gp

The TMDs in P-gp show significant diversity in their amino acid sequences reflecting the wide substrate-specificity throughout various organisms[48]. The NBDs, by contrast, share a substantial degree of amino acid sequence similarity among all classes of ABC transporters, whether of prokaryotic or eukaryotic lineage[49]. They contain the conserved motifs Walker A, Walker B, A-, Q-, H-loops, and the signature sequence (LSGGQ)[50,51]. Although translocation of drug substrates from the drug-binding site to the extracellular space requires the full functionality of all four constitute domains, a few single reactions are reported to occur in either of isolated TMDs and/or NBDs. At first, the two TMDs together are capable of binding drug substrate in absence of the NBDs[52]. In vivo drug-binding assays in HEK293 cells demonstrated the substrate-depending maturation of P-gp mutants by susceptibility for digestion by endoglycosidase H. Double mutants lacking both N- and C-terminal NBDs were able to mature only in the presence of various drug substrates (verapamil, vinblastine, or cyclosporine A) but failed to confer drug resistance upon drug efflux. This means that the NBDs and TMDs positively cooperate to mediate the drug transport process. Extrapolated from this, however, it is unclear whether drug transport is followed after ATP hydrolysis or ATP binding that initially occurs at the two NBDs.

The other partial reaction of the substrate transport process that is not dependent upon a complete assembly of the P-gp molecule is nucleotide binding at the NBDs. The characteristic motifs in the N- and C-terminal NBDs fold into nucleotide-binding pockets, with both domains contributing to two pockets to accommodate two nucleotides. The nucleotide (essentially ATP) is sandwiched between the Walker A, Walker B, B-, Q-, and H-loops of one NBD and the D-loop and signature sequence of the opposing NBD. This architecture, termed the 'ATP sandwich', was first predicted by modeling studies[53] based on the first high-resolution structure of the ATP-binding subunit[54]. Several biochemical and structural studies have demonstrated that isolated NBDs of ABC transporters exist as monomers with the nucleotide-binding sites in an open conformation in absence of nucleotide[55,56]. Addition of nucleotide resulted in the dimerization of the NBDs in a head-to-tail configuration. The binding of nucleotide is believed to stabilize the dimeric 'ATP sandwich' state by altering the electrostatic charge balance[55]. ATP-driven dimerization has been determined to have an activation energy of 60-70 kJ/mol[57,58], suggesting relatively large conformational changes. Mutation of a highly conserved glutamate in the Walker B domain of P-gp showed a severe impairment in ATP hydrolysis, thus obtaining a stable ATP-driven dimer[57,59-62]. The effect of such mutants (E556Q/E1201Q) was a tight and nonexchangeable occlusion of nucleotide (predominantly as adenosine triphosphate), alluded to as the "occluded nucleotide conformation"[62]. Locking the transporter in an ATP-bound conformation is agued to prevent ATP hydrolysis. The stoichiometry for the ATP-bound dimers (isolated NBDs)[55,63] was different than that for intact P-gp in the occluded ATP state[57,61]. This prompted the thought both are distinctive conformations.

The distinction between nucleotide-bound and occluded nucleotide conformations introduced new postulates regarding transition states of P-gp in the transport mechanism. Three distinct states have been speculated that result in the formation of the occluded nucleotide conformation: (1) an open dimer, (2) a symmetric closed dimer with ATPs bound at both NBDs, and (3) an asymmetrical occluded state where one of the two ATPs is tightly bound in a nonexchangeable form (Figure 5/reference 62 and 64 for reviews). The hypothesis that the occluded nucleotide conformation represents a reaction intermediate of the catalytic cycle impinges on earlier models describing ATP hydrolysis by ABC transporters (Figure 5 and Figure 6 for a continuation of the cycle). The alternative catalysis model (Senior *et al.*) states that the catalytic cycle of ATP hydrolysis alternates at the two

nucleotide-binding pockets at separate times[65]. This model emerged from a study wherein vanadate-induced nucleotide trapping at a single site was sufficient to yield full inhibition of drug transport by P-gp[66,67]. In this study, P-gp trapped nucleotides in the presence of vanadate plus Mg-ATP. Vanadate replaced the position of the *γ*-phosphate adjacent to ADP, thereby stably trapping ADP at either NBD. Reactivation of ATPase correlated with release of trapped ADP. Because ATPase activity was fully inhibited at a single catalytic site at either NBD, both nucleotide-binding sites are

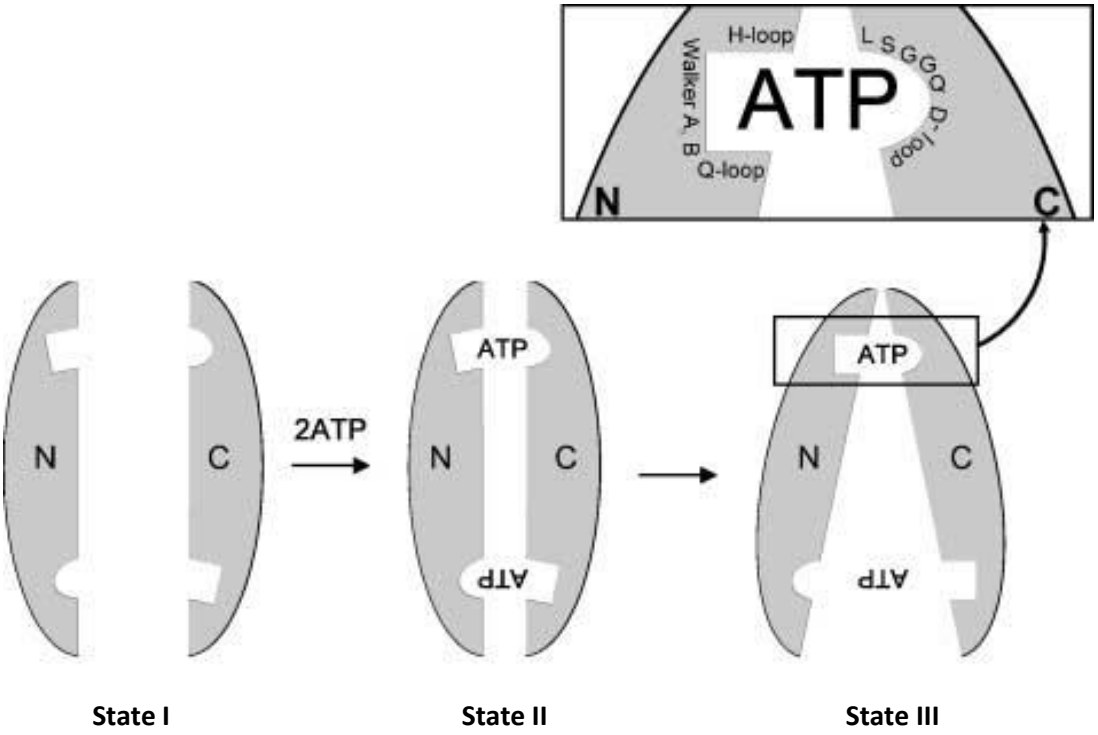


Figure 5: Schematic comparison of distinct transition states of the NBDs leading to the occluded nucleotide conformation. The open or inward-facing conformation (**State I**) is capable of binding two nucleotides at either catalytic site. Binding of ATP draws both NBDs together to form the symmetric closed dimer (**State II**). At this state the nucleotides are still exchangeable. In the mutated Walker B form, a transient intermediate is formed (**State III**) wherein one ATP is tightly occluded in an asymmetric conformation and cannot be exchanged. The rectangle depicts the ATP-binding site in state III more detailed, showing the recurring motifs among ABC transporters. This schematic view is based on the biochemical data with P-gp and structural information on isolated NBDs.

catalytically active and required for drug transport by P-gp. The same study showed that the nucleotide binding sites possessed a relatively low affinity in that a wide range of magnesium nucleotides were hydrolyzed, all with relatively high K_m values. More recently, the nonhydrolyzable ATP analogue ATP-*γ*-S showed a high affinity for P-gp as it was trapped in the asymmetric nucleotide conformation[68]. The occlusion of this analogue in wild-type P-gp showed comparable kinetics and thermodynamics for ATP occlusion in the Walker B glutamate (E556Q/E1201Q) mutant[57]. Other analogues, particularly 8-azido-[α-^{32}P]ADP, could not be used to generate the occluded nucleotide state in the Walker B (E556Q/E1201Q) mutant. This supported the postulate that the *γ*-phosphate is essential in driving dimerization of the N- and C-terminal NBDs. Therefore, ATP-*γ*-S was suitable as a nonhydrolyzable analogue when examining the translocation pathway of P-gp.

This study used the nucleotide-occlusion mediated P-gp arrest to monitor changes in drug-binding affinities during the transport process. To assess this, the radioactively labeled propafenone [^{3}H]GPV51 was utilized in photo-cross-linking assays. Incubation of P-gp in ATP-*γ*-S had a profound effect on substrate binding. The occluded nucleotide (ATP-*γ*-S) state of P-gp dramatically lowered the drug-binding affinity for non-radioactive GPV51 when compared to nucleotide-free P-gp, with

apparent K_d values of 128 µM and 42 µM respectively. Similarly, two separate studies using ATP-*γ*-S to arrest P-gp reported significant decreases in affinity for [^{3}H]vinblastine[69] and the conformation-sensitive monoclonal antibody UIC2[70]. Given that ATP-*γ*-S is similarly occluded in wild-type P-gp as the physiological substrate ATP is occluded in the (E556Q/E1201Q) mutant, one could carefully reason that ATP binding represents the step in the catalytic cycle of P-gp that induces the switch from low-affinity to high-affinity. However, the studies reviewed in this section made a sharp distinction between the ATP-bound and the occluded nucleotide conformation of P-gp (Figure 5) with the latter state causing the switch in substrate affinities. It is difficult to assess whether the ATP-bound could do so either in wild-type P-gp as this conformation cannot be arrested due to immediate ATP hydrolysis. Nevertheless, it is less likely that ATP hydrolysis converts the high affinity conformation into the low affinity conformation.

Finally, the occlusion of ATP most probably precedes ATP hydrolysis. The products of ATP hydrolysis (ADP plus P_i) have been shown by several studies to destabilize the nucleotide sandwich dimer, leading to dissociation. The dissociation is due to electrostatic repulsions between the ADP product bound to the Walker A motif and the inorganic phosphate (P_i) bound to the LSGGQ signature motif in the apposing nucleotide-binding site of the NBD[44,55,71]. By extension, the inorganic phosphate is known to exhibit very low affinity for the signature sequence motif of P-gp. Agreeing the alternative catalysis model, the ADP bound to one catalytic site dissociates while another ATP occludes at the second site. This is accompanied by a conformational change, yielding the closed 'nucleotide sandwich' dimer (Step IV in Figure 6).

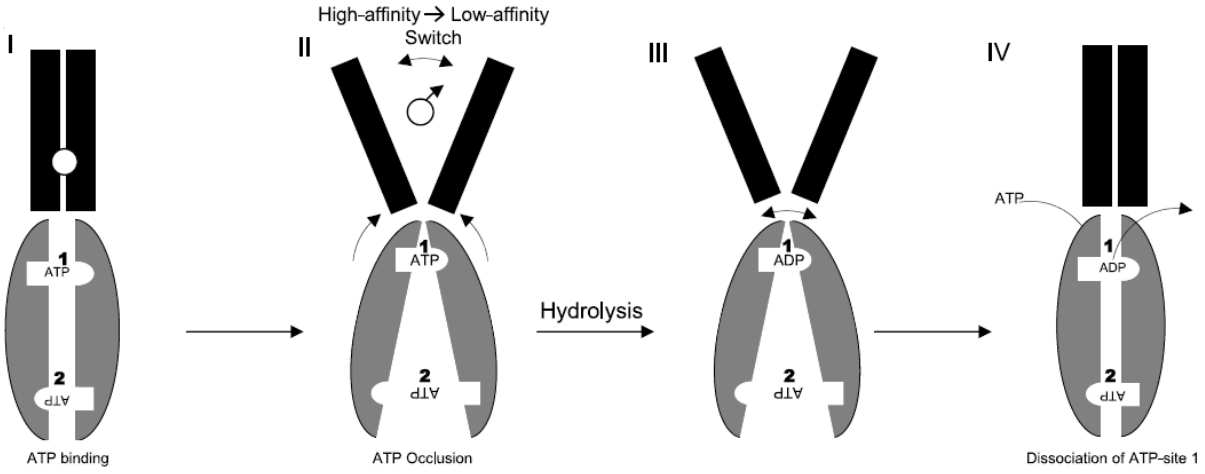


Figure 6: Schematic mechanism of the catalytic cycle of ATP hydrolysis including the formation of the occluded nucleotide conformation. Black rectangles) TMDs; gray ovals) NBDs; white circle) transport substrate. For simplicity reasons, the inorganic phosphate (P_i) is left out. The two nucleotide-binding sites of the NBDs are labeled. The box in figure 5 gives a detailed illustration of the nucleotide-binding sites in the occluded state. With the exception of the drawn TMDs, step I and II in this figure are equal to state II and III in figure 5. Binding of ATP at site 1 and 2, concurrent to binding of substrate, induces the dimerization of the NBDs (**I**). The occluded nucleotide conformation is then rapidly followed. Occlusion of one ATP molecule at site 1 causes a conformational change that is transmitted to the TMDs and causes the low affinity to high affinity switch, followed by exposure of the transport substrate to the extracellular space (**II**). The occluded ATP at site 1 is hydrolyzed to yield ADP which results into dissociation of the apposing subunits (**III**). After dissociation of ADP from site 1 this site is occupied by another ATP, resetting the nucleotide-bound conformation ready to bind substrate (**IV**).

Interdomain cross-talk mediates drug transport

Like all ABC transporters, P-gp uses the energy from ATP hydrolysis to efflux cytotoxic drug substrates against a concentration gradient. Consequently, substrate-binding must elicit an effect on the ATP catalytic domains if it is to be outwardly transported. In the previous section we adequately discussed the reaction intermediates in the catalytic cycle of P-gp, incorporating transition states into developing mechanistic models. We also noticed the functional independency of both TMDs and NBDs in taking part of these transition states, i.e. nucleotide occlusion in isolated NBDs. Here, we shall further enlighten the cooperation between the TMDs and NBDs in conveying outward translocation of substrates.

Previously, the mechanism by which drug transport is coupled to ATP hydrolysis was studied using several strategies. Usually a variety of compounds are tested for some of them are known to either stimulate or inhibit ATPase activity, e.g. nontransported modulators. The basal rate of ATP hydrolysis is stimulated by the binding of compounds such as verapamil, vinblastine, and rhodamine 123[72-74]. Further evidence of direct conformational coupling between the drug-binding sites and ATP catalytic domains came from allosteric alterations in the fluorescence spectrum of MIANS, a fluorescent probe[75]. Binding of several drugs and chemosensitizers to MIANS-labeled P-gp caused substantial quenching of the probe fluorescence within the NBDs. More specifically, MIANS was labeled at two conserved cysteines within the Walker A motifs of each NBD[76].

A study conducted by Loo *et. al*. revealed a relatively close proximity between the LSGGQ in one NBD and the Walker A motif in the other NBD[77]. Later, Loo *et al*. (2003) studied a possible role for the LSGGQ signature sequence in transmitting conformational changes to the nucleotide-binding sites in the NBDs[78]. In approaching this, the effect of drug substrates on cross-linking between the LSGGQ and Walker A motifs was evaluated. To enable cross-linking between either motifs, two mutants were constructed that both had one endogenous cysteine. L531C/Cys^{1074} contained a cysteine built in the N-terminal ^{531}LSGGQ^{535} motif (L531C) and an endogenous cysteine (Cys^{1074}) in the C-terminal Walker A site. The second mutant (Cys^{431}/L1176C) contained the endogenous Cys^{431} in the N-terminal Walker A motif and a cysteine built in the C-terminal ^{1176}LSGGQ^{1180}. Transport assays in P-gp containing membranes and subsequent immunoblot analysis revealed different cross-linking rates for stimulatory and inhibitory substrates. Binding of several stimulatory substrates, including calcein-AM and verapamil, each increased the rate of cross-linking upon ATP-driven dimerization of the cysteine containing NBDs. In other words, the LSGGQ motif and Walker A site move closer together. Conversely, incubation with inhibitory substrate caused a strongly delayed cross-linking due to a greater separation between the apposing cysteines in the LSGGQ motif and the Walker A site. These results indicated that ATPase activity can either positively or negatively regulated by binding of stimulatory or inhibitory drug substrates, respectively. Hence, drug-binding must trigger long-range conformational changes that are transmitted to the NBDs.

Recently, the same team adopted a similar cross-linking approach to further reinforce their evidence in the dependency of ATPase activity on the distance between the two ATP catalytic domains (NBDs)[79]. The use of a short (8 Å) cross-linker, in fact, demonstrated this dependency is strongly proportional. Cysteines were introduced in Cys-less human P-gp at locations close to the LSGGQ and Walker A sites of NBD1 and NBD2 respectively. Subsequently, verapamil-transport led to an enhanced (>10-fold) basal ATPase activity. However, the addition of the short cross-linker (M4M) resulted in a 14-fold higher ATPase activity upon the same verapamil stimulation. These results indicated that trapping of NBD1 and NBD2 in close proximity by covalent linkage (M4M) mimics activation of ATPase activity as accomplished by binding of transport substrates. Therefore, this cross-linking assay provides a tool to illustrate the importance of the closed conformation in stimulating ATP hydrolysis.

Due to little homology among the TMDs of ABC transporters less is known about interactions between these regions. Therefore, the examination of domain-domain interactions was further expanded by including interactions between TM segments. Previous disulfide cross-linking studies and labeling of cysteine mutants with thiol-reactive drug substrates suggested TMs 4-6 of TMD1 and TMs 9-12 of TMD2 contribute largely to the drug-binding pocket at the interface of the homologous

P-gp halves[80-83]. Transmembrane segments TM6 (TMD1) and TM12 (TMD2) are directly connected to their respective NBD, making both helices intimately involved in conformational changes enabling cross-talk between drug-binding and ATPase activity[84]. Oxidative cross-linking was used to monitor structural rearrangements between TM6 and TM12 induced by drug-stimulated ATPase activity. To this end, six pairs of cysteines were introduced into a Cys-less mutant of P-gp to allow generation of mutant residue pairs in TM6 and TM12. A previous study showed that Cys-less P-gp exhibits activity that is comparable with that of the wild-type transporter. Therefore, endogenous cysteines have no apparent functional relevance in binding and transporting substrates. Addition of substrates and ATP affected cross-linked mutant pairs differently. Cross-linking between L332C and L975C occurred during ATP hydrolysis rather than ATP binding as nonhydrolytic conditions by ATP analogues and nucleotide trapping induced by vanadate did not result into cross-linking. Conversely, the effect of cross-linking between TM6 and TM12 on drug-stimulated ATPase activity was tested and whether inhibition, if occurred, could be reserved by the reducing agent dithiothreitol (DTT). As the mutant P350C/S993C exhibited the highest degree of cross-linking it showed a dramatic reduction in verapamil-stimulated ATPase activity (±75%). Recovery of the activity was achieved upon addition of DTT as this allegedly broke the disulfide bond of the P350C/S993C mutant and allowed freely moving TM6 and TM12. Among other drug substrates used in the individual experiments, verapamil and vinblastine were most efficient in stimulating ATPase activity.

Together, these results indicate TM6 and TM12 are essential for cross-talk between drug-binding and ATPase activity. The direction in which the communication proceeds is still disputable. Cross-linking was most profound between L332C and L975C upon ATP hydrolysis rather than binding. This finding evidently manifests that ATP hydrolysis endeavors repacking of the TM segments in both TMDs. Additionally, this observations suggest conformational communication from the catalytic NBDs to the drug-bound pocket. However, the strong cross-linking of the P350C/S993C mutant distally inhibited ATPase activity, thereby reducing ATP hydrolysis. This suggests cross-talk proceeds in the opposite direction, namely from the drug-binding site to the NBDs in the ATP-binding sandwich. On the other hand, given that binding of drug substrate and nucleotide can occur randomly, the ATPase activity can be compromised prior to verapamil-binding and is merely due to cross-linking of the P350C/S993C mutant. In congruence with this, a recently published article by the same authors states that P-gp exhibits basal ATPase activity in absence of drug substrate[79]. This is characterized by cycling between the closed (open to extracellular space) and closed (open to cytoplasm) conformations of P-gp. Hence, cross-linking between the same mutant residues in ligand unbound P-gp is predicted to arrest this cycle in the closed conformation (nucleotide-binding competent).

CHARACTERIZATION OF THE DRUG-BINDING SITES

Refining the drug-entry portals of P-gp

The great majority of mechanistic and structural features occurring coordinately at the TMDs and NBDs of P-gp are essential for drug transport and, thus, conserved among all ABC transporters. Translocation of any transportable compound through any type of ABC transporter requires the ATP-driven dimerization of the NBDs and repacking of TM segments, irrespective of how these central events are precisely coupled. However, despite P-gp is duplicated in homologous parts, the two domains constituting each half seem to have different evolutionary backgrounds. In contrast to the NBDs, the TMDs show a loss of evolutionary conservation throughout a large number of organisms, reflecting the poor overlap of substrate specificity profiles among diverse species. In addition, these structural units participate in the formation of multiple drug-binding sites that recognize a multitude of structurally unrelated compounds. The remainder of this essay will be devoted mainly to current

findings on the characterization of the drug-binding sites in the drug-binding pocket and how they interact with and distinguish between substrate drugs and modulators. These studies attempted to gain more insight in the substrate poly-specific nature of P-gp. In addition, structures that contribute to the stability of the whole protein structure will be presented.

Molecular dynamics (MD) simulations have proven valuable as a tool to model the behavior of various compounds inside the common drug-binding pocket by analyzing the type and number of established contacts with certain residues. In addition, this method allows to examine transmembrane protein function in a broader context with its lipid environment which is impossible for crystallographic structures. Recently, MD simulations were used to further refine the crystal structure of mouse P-gp[18] by building the missing linker sequence and adding the lipid bilayer membrane[20]. To assess the importance of these structures, the protein was first inserted into membranes enriched for POPC lipids to minimize deviations from the initial insertion angle[85]. Additionally, POPC lipids are naturally present in eukaryotic cell membranes.

The linker sequence was designed on the basis of secondary structure prediction programs. These and more exhaustive programs predicted that 87.72% of the residues were exposed with a probability for the occurrence of α-helices and β-sheets of only 10.53% (Figure 7). This is consistent to the highly charged and flexible nature of the linker region as predicted in earlier studies[86,87]. Also, sequences were predicted that favor protein-protein interactions and protein-nucleotide interactions. A recent study using limited proteolysis on the linker region shed light on its involvement in ATPase activity[87].

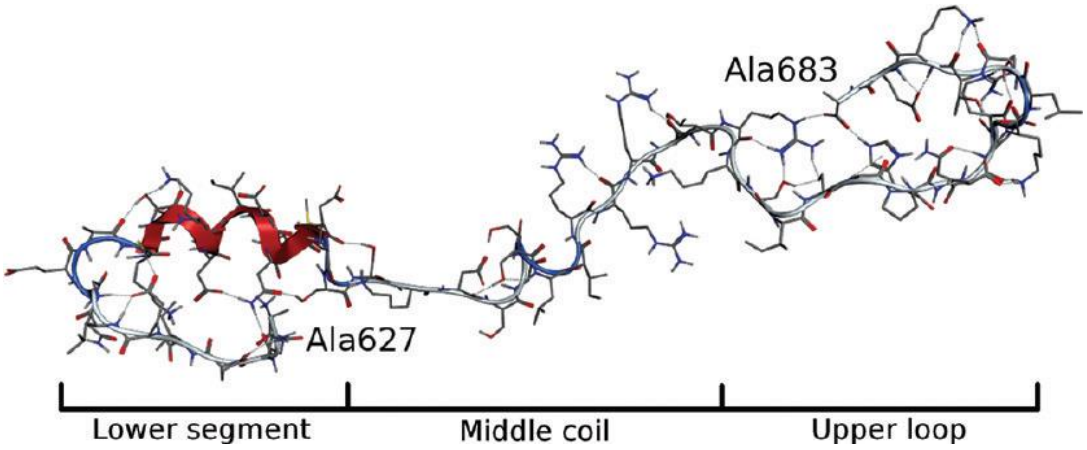


Figure 7: Model of the predicted linker secondary structure. The model depicts the high number of exposing residues (87.72%). The predicted α-helix is shown (lower segment) as well as the N-terminal Ala627 and the C-terminal Ala683 residues. Although not shown here, the linker sequence is situated between the two NBDs.

Simulations clearly indicated that the linker sequence, together with the POPC-enriched lipid bilayer, greatly decreased the distance variations between the two NBDs. The flexibility of this secondary structure apparently functions as an intermediary between the two domains, thereby increasing the stability of the cytoplasmic portion of P-gp. These results support the fact that both the linker region and the membrane contribute largely to the stability of the transporter's entire structure. Moreover, only one accessible portal in the DBP was detected in the inner leaflet and was formed by TMD 10/12. The other portal, formed by TMD 4/6, remained closed during the entire simulation. A similar block of the lateral opening was also found in the crystal structure of *C. elegans* (section 'Structure-function relationships of P-gp').

The refined and completed mouse P-gp model was then used to assess the type and number of protein-ligand interactions by testing various compounds that were known as modulators and/or substrates (e.g. verapamil, vinblastine, and tariquidar). Because many P-gp substrates are known to favor partition into the membrane, the authors postulated that a different number of interactions inside the drug-binding pocket may be a determinant for distinguishing modulators and substrates. Previous studies, furthermore, identified hydrophobic and hydrogen-bond acceptor groups as responsible for the majority of interactions inside the drug-binding pocket[88-91]. Differences between modulators and substrates registered in this study revealed that all modulators tested exhibited a high number of nonbonded interactions (e.g. vdw interactions) when compared with substrates. These nonbonded interactions were governed by aromatic residues. Concerning the number of interactions, the data showed that modulators frequently establish a higher number of interactions that occurred simultaneously (a table listing interactions with all modulators and substrates is provided in reference 20). Eventually, this characteristic was chosen as the major determinant in illustrating the difference between modulators and substrates of P-gp. From this one could carefully classify modulators into groups of molecules that harbor a relatively great number of simultaneous interactions with residues lining the drug-binding site. The following will further discuss the characterization of drug-binding sites and classifications for modulators and substrates.

P-gp efflux modulation and drug promiscuity

The examination of protein-ligand interactions in mouse P-gp was resumed with a series of molecular docking computations for an in-depth characterization of previously identified drug-binding sites[21]. In addition, a classification model was created that enabled discrimination between modulators and substrates. Two of the three drug-binding sites targeted for this extensive analysis were initially proposed during a study in the late 1990s (Shapiro and Ling)[92]. Both sites positively cooperated with each other and were named the H-site and the R-site due to the distinct drug specificities registered for Hoechst 33342 and rhodamine-123, respectively. More functional features of these compounds and their respective binding sites were unwrapped in several successive studies. For instance, it was shown that both compounds could be effluxed from the membrane inner leaflet to the outer leaflet through both sites, supporting the 'hydrophobic vacuum' cleaner model[93]. In congruence with that, a recent study confirmed the existence of two translocation pathways[94]. In a different study, Shapiro and Ling proposed a third drug-binding site with a positive allosteric effect on both R- and H-sites[95].

Despite all crucial contributions from these studies (some of which not included here) the H- and R-sites remain largely uncharacterized namely in its amino acid composition and physicochemical properties. Also, the specific locations of both drug-binding sites have not been entirely identified. The docking study, reviewed here, is the latest attempt to elucidate these drug-binding sites. The objective was to fully define the lining and location of the residues constituting the three drug-binding sites described above. Also, the authors proposed a classification scheme to categorize modulators and substrates separately on the basis of differing protein-ligand interactions. Due to space restrictions posed on the length of this essay, a greater portion will be devoted to the generation of the classification scheme shall be discussed.

In order to map all detectable protein-ligand interactions more extensively, the authors used a database of 68 molecules (substrates, modulators, efflux probes, and lipids) to assess their docking abilities into the drug-binding pocket. The three main drug-binding sites in P-gp above-mentioned were proposed and (in part) identified by experiments with verapamil, Hoechst 33342, and rhodamine-123. In this study, top-ranked docking poses of these and additional substrates in the DBP determined and validated the assignments of the H-, R-, and M- sites. The ranking was based on poses of compounds that bound P-gp with the lowest free energy. The M-site (modulator) was defined by docking poses of verapamil. Docking of this compound occurred at the top of the cavity (DBP) next to the outer leaflet, marking this as the M-site location (Figure 8). The R-site was assigned mainly by top-ranked docking poses of rhodamine-123, a major drug substrate of P-gp. The data

supported a localization of this site near the cytoplasmic inner leaflet (Figure 8). The H-site was identified by top-ranking poses of Hoechst 33342. Docking of additional molecules was analyzed to validate these assignments of the H-site as well as the R-site. Both drug-binding sites were predicted to be buried in the cytoplasmic (inner) leaflet of the membrane at separate positions: 2.4 Å for the water/lipid interface (H-site) against 0.5 Å mapped for the R-site (Figure 8). The H- and R-site were preferentially bound by drug substrates. Moreover, the docking studies enabled the identification of additional drug-binding site properties, including residue distribution, mean polarity, and volume. The M-site was lined predominantly with aromatic residues, making this the most hydrophobic site compared to the H- and R-site. In addition, the M-site was predicted to be the smallest site with a volume 30 to 39% lower than the R-site and H-site, respectively.

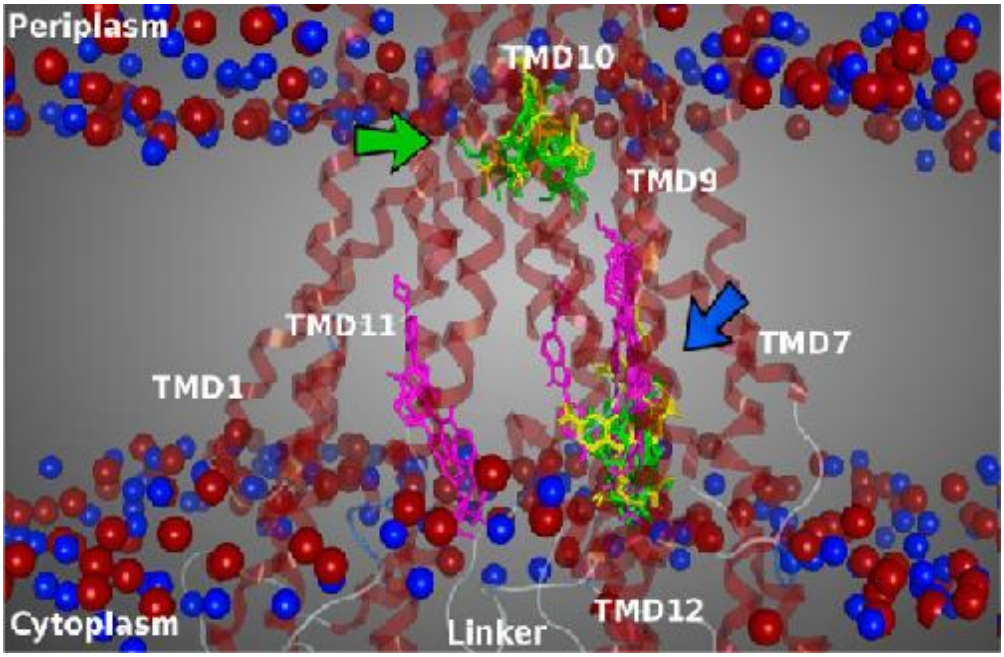


Figure 8: The locations of the three drug-binding sites in the DBP were determined by top-ranked docking of three molecules. Top-ranked docking poses for verapamil (green), rhodamine-123 (yellow), and Hoechst 33342 (pink). Corresponding binding sites for the M-site and R-site are marked by green and blue arrows, respectively. A separate figure (not shown) was used to indicate the H-site with a red arrow pointing (in this figure) at Hoechst 33342. However, in the separate figure different compounds in their best-ranked docking poses were drawn than the ones drawn in the figure above. Red and blue spheres represent the lipid headgroups of the membrane inner (bottom) and outer (top) leaflet.

In general, a considerable number of compounds exhibit efflux modulation effects on other molecules in the DBP. To evaluate the modulation ability of certain compounds, the affinity of several probe substrates for the drug-binding sites was determined. For instance, daunorubicin (anticancer drug) and colchicine (natural product from plants) demonstrated an effect on rhodamine-123 efflux. The binding free energies of daunorubicin and rhodamine-123 were -10.2 kcal.mol^{-1} and -9.2 kcal.mol^{-1}, respectively. As a consequence, binding of daunorubicin at the R-site may be favored over that of rhodamine-123 which decreases the efflux rate of the latter. Therefore, daunorubicin works as a competitive inhibitor depending on its concentration. Conversely, colchicine has a high binding free energy (-8.4 kcal.mol^{-1}) compared with that of rhodamine-123, making the R-site competition favorable for rhodamine-123. At its turn, this effect displaces colchicine in a separate translocation pathway as was also observed in similar experiments by Shapiro and Ling[92]. Remarkably, promiscuous behavior among many compounds has been reported, e.g. for verapamil

and vinblastine. Both molecules are occasionally described as both substrate and modulator. The experiments described here indicated that the best-ranking docking poses of verapamil were positioned at both the M-site and H-site. This promiscuity can be explained by the identical binding free energies at these sites. Moreover, additional docking experiments were performed to map efflux modulation by several other molecules (results of which not summed here). Altogether, the docking results allowed the identification of hydrogen-bond and nonbonded interactions between both homologous halves of P-gp, agreeing the observations from MD simulations discussed earlier. The docking results presented here, however, indicated that these interactions were exclusively localized to the M-site. This may provide an explanation of the frequently observed modulation effects that also known drug substrates seem to exhibit.

All findings inspired the generation of a classification scheme to distinguish modulators from substrates. Selection criteria used were docking free energies and cross interaction capacities at the M-site. Ranked according an increasing affinity for the M-site, the molecules in the docking database were categorized as follows: (a) nonsubstrates; (b) transported substrates; (c) nontransported substrates; and (d) modulators. Because the binding energy formed the main determinant in scoring docking poses of candidate compounds, a defining boundary was set to distinguish nonsubstrates from molecules in all other categories. Therefore, an empirical cutoff of -7.0 kcal.mol^{-1} above which molecules were classified as nonsubstrates. These molecules are not likely to interact with P-gp at all.

Transported substrates showed an increased affinity (lower ΔG) for the substrate-binding H- and R-sites, with seldom cross interaction with the M-site. Of the 32 substrates contained in the database, nine molecules (including doxorubicin, etoposide, and Hoechst 33342) docked exclusively in both substrate-binding sites (H and R) with binding energies between -10.5 and -9.0 kcal.mol^{-1}. Several additional molecules registered at least one pose at the M-site with weak cross interaction. Other molecules docked either exclusively at the R-site (methotrexate) or at the H-site (cyclosporine A). Of note, methotrexate (MTX) is known to have an active carrier-mediated influx that increases its intracellular concentration. However, MTX is actively effluxed by P-gp in carrier-deficient cells which were used for the docking studies discussed here.

Nontransported substrates exhibited a slightly higher preference to dock at the M-site with weak cross interaction for the H- and R-sites. Six molecules showed lower binding free energies for the M-site between -9.1 kcal.mol^{-1} (itraconazole) and -7.9 kcal.mol^{-1} (QZ59-RRR). Within this class, differences in P-gp's stereospecificity were observed. A stronger cross interaction was found for QZ59-SSS (IC_{50} = 1.2 μM) than for stereoisomer QZ59-RRR (IC_{50} = 4.8 μM). Although colchicine is considered a P-gp substrate, the classification model registered docking poses of this molecule at the M-site which indicates cross interaction capability for this compound. Additionally, itraconazole had identical binding energies for both the H-site and M-site enabling moderate cross interactions for these sites. Overall, the increased affinity for docking at the M-site with moderate cross-interaction for the R- and H-site is consistent with promiscuous behavior. For this reason, a fraction of the molecules classified as nontransported substrates could be argued to be modulators as well.

Modulators had a lower ΔG and exhibited a clear preference to the M-site. Of the 19 modulators contained in the total database, eight were eventually grouped as modulators in the classification scheme and had binding energies ranging from -7.9 kcal.mol^{-1} up to -11.5 kcal.mol^{-1}. The lower limit was registered for paclitaxel. This compound is frequently described in literature as a P-gp substrate although a number of studies identified paclitaxel as a potential modulator on several cell lines[96-98]. Amprenivar (IC_{50} = 8.6 μM)[99] had a preference to dock at both P-gp halves, with a slightly lower binding free energy at the M-site (-8.9 kcal.mol^{-1}) than registered at the R-site (-8.5 kcal.mol^{-1}).

These docking results have indicated a clear increase in cross interaction capability from transported substrates to nontransported substrates, displayed by a progressive shift from substrate-binding sites (R- and H-sites) to the M-site (lower ΔG values). Moving further to the modulator group, cross interaction among the M-site and substrate-binding sites occurs even more frequently, accompanied by further decrease in binding free energies (registered minimum of -11.5 kcal.mol^{-1}). These docking studies evidently showed that P-gp efflux modulation can be achieved upon direct competition with molecules at the substrate-binding sites as a consequence of conformational

impairment at the M-site or both. Finally, competitive binding, as demonstrated by these docking studies, suggests an internal cavity volume that is competent for accommodation of at least two molecules. Cavities with particular volumes have been suggested in previous studies[29].

SUMMARY

- Mouse and *C. elegans* P-gp crystallize each as two homologous halves in a cytoplasmic-facing conformation when unbound to nucleotide. Each P-gp half comprises one TMD (6 TM helices) and one cytoplasmic NBD.
- The drug-entry portals are lateral openings lined by TM helices containing predominantly hydrophobic and aromatic residues. The portals are asymmetrically situated with respect to each other with only one portal allowing entrance of primarily membrane-partitioning drugs from the membrane inner leaflet.
- Binding of nucleotide in the open (cytoplasmic-facing) conformation drives the dimerization of both NBDs into an 'nucleotide-bound sandwich' which, at its turn, triggers initial repacking of the TM helices that alter drug-binding affinities. Occlusion of the nonhydrolyzable nucleotide ATP-γ-S introduces an asymmetric transition state that precedes ATP hydrolysis.
- The energy released from ATP-hydrolysis is coupled to a conformational change (tail-to-head) that is associated with a high-affinity to low-affinity switch at the drug-transport pathway. Occlusion of the nonhydrolyzable ATP-γ-S significantly compromises drug-binding.
- Vanadate-induced trapping of ADP at a single nucleotide-binding site fully inhibits P-gp activity suggesting the catalytic cycle of P-gp requires ATPase activity from both NBDs.
- Several compounds show promiscuous behavior by their shared affinity for binding sites that favor substrates (R/H-sites) as well as sites that favor binding of modulators (M-sites). This kind of cross interaction at distinct drug-binding sites is frequently associated with transportable substrates having identical binding free energies for either sites. Molecules classified as modulators

DISCUSSION AND PERSPECTIVES

Unveiling the poly-specific nature of P-glycoprotein in drug efflux is crucial in finding potential solutions to multidrug resistance phenotypes that frequently arise in many tumorous cell lines. The experimental studies summed above contributed to new concepts and insights into structural and mechanistic features underlying poly-specific drug transport by P-gp. However, these and other studies also provided sufficient experimental information that needs to be clarified to further elucidate the remarkable drug promiscuity of this transporter. Also, a mechanistic model of drug efflux that incorporates each step of the complete catalytic cycle is not available in literature.

P-gp is able to efflux a vast array of structurally unrelated molecules. Substrate poly-specificties of such measures probably require binding affinities of drugs that lie close to one another for if binding of each drug molecule was highly specific a multitude of unique drug-binding-sites would have been distributed throughout the common drug-binding pocket. The series of docking studies presented above support the view of shared drug-binding affinities as most notably demonstrated by the capability of cross interaction among the three identified drug-binding sites. The models extracted from these docking studies also show a clear competition of drugs for a particular binding site due to slight differences in binding free energies. This directly indicates the internal cavity volume of P-gp in the cytoplasmic-facing conformation has a volume that is large enough to accommodate several molecules at once (Figure 8).

Additional evidence of lowered drug affinities linking to substrate poly-specificity by P-gp is drawn from experiments wherein drug transport capacities by *C. elegans* P-gp was studied in context with its direct lipid environment. Transport assays were used to compare drug-stimulated ATPase activity in detergent-purified P-gp and overexpressed in biological membranes. Stimulation of ATPase activity by actinomycin D and paclitaxel was drastically elevated in membranes when compared to detergents. This supported the postulate that the majority of P-gp transport substrates partition in the membrane (inner leaflet) rather than residing in the cytoplasm. Membrane-partitioning of drug substrates is usually associated by relatively high local concentrations of the particular compounds. Transport by P-gp depending on these circumstances implies that the intrinsic affinity of P-gp for membrane residing substrates is rather low, agreeing the conclusions drawn from the docking studies.

On the other hand, the docking studies and transport assays above-described do not sufficiently account for the conformational alterations of the TM segments through dimerization of the NBDs. The experiments discussed also in this essay demonstrate a clear shift in drug-binding affinity upon locking P-gp in the occluded-nucleotide conformation. This provides evidence that initial repacking events of TM segments is induced by ATP-binding at the catalytic NBDs. Thus conformational cross-talk between both constitute domains of P-gp alters the binding affinities of drug substrates through subtle repacking of the TM helices. It is reasonable to argue that these alterations lead to introductions of entirely novel drug-binding sites and, thus, shifts the affinity of P-gp for one compound to the next. Procedural events at the NBDs like ATP hydrolysis and ATP-binding at the alternate pocket may induce more dramatic rearrangements of the TM segments, shifting binding affinities of drug substrates. This theory provides a more dynamic basis for the poly-specific drug transport of P-gp.

Looking out at future aims, it is essential to address such hypothesis with appropriate experiments. The crystallographic data for mouse and *C. elegans* P-gp provided a framework for additional studies on P-gp function already conducted. However, a more conclusive analysis of the structure-function relations requires structure determinations at higher resolutions (< 2.0 Å). For instance, a cocrystal of P-gp or isolated NBDs complexed with ATP-γ-S could be grown to examine whether the occluded-nucleotide conformation genuinely represents a transition state of the ATPase catalytic cycle. Moreover, MD simulations and docking studies may provide a more detailed demonstration of the conformational coupling between the NBDs and TMDs. These studies will contribute to a more detailed picture of the mechanistic model of drug efflux and analyze the dynamic behavior of the TM segments upon nucleotide-binding.

REFERENCES

1. 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. *J. Mol. Biol.* 293(2), 381-99.

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

3. Gerlach, J.H., Endicott, J.A., Juranka, P.F., Henderson, G., Sarangi, F., Deuchars, K.L., Ling, V. (**1986**). Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. *Nature.* 324(6096), 485-9.

4. Gros, P., Croop, J., Housman, D. (**1986**). Mammalian multidrug resistance gene: complete cDNA

sequence indicates strong homology to bacterial transport proteins. *Cell*. 47(3), 371-80.

5. Ramachandra, M., Ambudkar, S.V., Chen, D., Hrycyna, C.A., Dey, S., Gottesman, M.M., Pastan, I. (**1998**). Human P-glycoprotein exhibits reduced affinity for substrates during a catalytic transition state. *Biochemistry*. 37(14), 5010-19.

6. Lam, F.C., Liu, R., Lu, P., Shapiro, A.B., Renoir, J.M., Sharom, F.J., Reiner, P.B. (2001). beta-Amyloid efflux mediated by p-glycoprotein. *J. Neurochem*. 76(4), 1121-28.

7. Giacomini, K.M., Huang, S.M., Tweedie, D.J., Benet, L.Z., Brouwer, K.L., Chu, X., Dahlin, A., Evers, R., Fischer, V., and Hillgren, K.M., et al.; International Transporter Consortium (**2010**). Membrane transporters in drug development. *Nat. Rev. Drug. Discov*. 9, 215-36.

8. Fromm, M.F. et al. (**2002**). The influence of MDR1 polymorphisms on P-glycoprotein expression and function in humans. *Adv. Drug. Deliv. Rev*. 54, 1295–1310.

9. Marzolini, C. et al. (**2004**). Polymorphisms in human MDR1 (P-glycoprotein): recent advances and clinical relevance. *Clin. Pharmacol. Ther.* 75, 13–33.

10. Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I., Willingham, M.C. (**1987**). Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc. Natl. Acad. Sci. USA.* 84(21), 7735-8.

11. Fromm, M.F. (**2004**). Importance of P-glycoprotein at blood-tissue barriers. *Trends Pharm. Sci.* 25(8), 423-29.

12. Schinkel, A.H. et al. (**1994**) Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* 77, 491–502.

13. Schinkel, A.H. et al. (**1996**) P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *J. Clin. Invest*. 97, 2517–2524

14. Higgins, C.F. (**1992**). ABC transporters from microorganisms to man. *Annu. Rev. Cell Biol*. 8, 67–113.

15. Suttana, W., Mankhetkorn, S., Poompimon, W., Palagani, A., Zhokhov, S., Gerlo, S., Haegeman, G., Berghe, W. v.d. (**2010**). Differential chemosensitization of P-glycoprotein overexpressing K562/Adr cells by withaferin A and Siamois polyphenols. *Molecular Cancer*. 9(99), 1-22.

16. Santos, A. and Paulo, A. (**2013**). Small Molecule Inhibitors of Multidrug Resistance Gene (MDR1) Expression: Preclinical Evaluation and Mechanisms of Action. *Current cancer drug targets*. ISSN: 1873-5576.

17. Hughes, J., Crowe, A. (**2010**). Inhibition of P-glycoprotein-mediated efflux of digoxin and its metabolites by macrolide antibiotics. *J. Pharmacol. Sci*. 113(4), 315-24.

18. Aller, S.G., Yu, J., Ward, A., Weng, Y., Chittaboina, S., Zhuo, R., Harrell, P.M., Trinh, Y.T., Zhang, Q., Urbatsch, I.L., Chang, G. (**2009**). Structure of P-Glycoprotein Reveals a Molecular Basis for Poly-Specific Drug Binding. *Science*. 323, 1718-22.

19. Jin, M.S., Oldham, M.L., Zhang, Q., Chen, J. (**2012**). Crystal structure of the multidrug transporter P-glycoprotein from *Caenorhabditis elegans*. *Nature*. 490, 566-70.

20. Ferreira, R. J., Ferreira, M. J. U., dos Santos, D. J.V. A. (**2012**). Insights on P-glycoprotein's efflux mechanism obtained by molecular dynamics simulations. *J. Chem. Theory Comput*. 8, 1853−64.

21. Ferreira, R. J., Ferreira, M. J. U., dos Santos, D. J.V. A. (**2013**). Molecular Docking Characterizes Substrate-Binding Sites and Efflux Modulation Mechanisms within P-Glycoprotein. *J. Chem. Info. Mod.* xxx, xxx-xxx.

22. Loo, T. W., and Clarke, D. M. (**1995**). Membrane Topology of a Cysteine-less Mutant of Human P-glycoprotein. *J. Biol. Chem*. 270, 843-48.

23. Kast, C., Canfield, V., Levenson, R., 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), 4402-11.

24. 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 Lett.* 580, 1049-55.

25. 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. *EMBO J.* 1(8), 945–51.

26. Higgins, C.F., and Linton, K. J. (**2004**). The ATP switch model for ABC transporters. *Nat. Struct. Mol. Biol.* 11, 918-26.

27. 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. *J. Biol. Chem*. 272(16), 10685-94.

28. Lee, J.Y., Urbatsch, I.L., Senior, A.E., and Wilkens, S. (**2008**). Nucleotide-induced structural changes in P-glycoprotein observed by electron microscopy. *J Biol Chem*. 29;283(9), 5769-79.

29. 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. *J. Biol. Chem*. 278(41), 39706-10.

30. Crowley, E., O'Mara, M.L., Reynolds, C., Tieleman, D.P. Storm, J., Kerr, I.D., Callaghan, R. (**2009**). Transmembrane helix 12 modulates progression of the ATP catalytic cycle in ABCB1. *Biochemistry* . 48, 6249−58.

31. Rothnie, A., Storm, J., Campbell, J., Linton, K. J., Kerr, I. D., Callaghan, R. (**2004**). The coupling mechanism of P-glycoprotein involves residue L339 in the sixth membrane spanning segment. *J. Biol. Chem*. 279, 34913−34990.

32. Schumacher, M.A. and Brennan, R.G.(**2002**). Structural mechanisms of multidrug recognition and regulation by bacterial multidrug transcription factors. *Mol. Mircobiol*. 45(4), 885-93.

33. Chen, Y.J., Pornillos, O., Lieu, S., Ma, C., Chen, A.P., and Chang, G. (**2007**). X-ray structure of EmrE supports dual topology model. *Proc. Natl. Acad. Sci. USA*. 104(48), 18999-9004.

34. Loo, T.W., Bartlett, M.C. and Clarke, D.M. (**1997**). Identification of Residues in the Drug-binding Site of Human P-glycoprotein Using a Thiol-reactive Substrate. *J. Biol. Chem*. 272(51), 31945-8.

35. 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. *Biochem. J*. 399, 351-59.

36. Higgins, C.F. and Gottesman, M.M. (**1992**). Is the multidrug transporter a flippase? *Trends Biochem. Sci.* 17, 18–21.

37. Gatlik-Landwojtowicz, E., Aanismaa, P. & Seelig, A. (**2006**). Quantification and characterization of P-glycoprotein-substrate interactions. *Biochemistry.* 45, 3020–32.

38. 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. *Proc. Natl. Acad. Sci. USA*. 104, 19005–19010.

39. Screpanti, E. and Hunte, C. (**2007**). Discontinuous membrane helices in transport proteins and their correlation with function. *J. Struct. Biol*. 159, 261–67.

40. Oldham, M.L., Davidson, A.L. and Chen, J. (**2008**). Structural insights into ABC transporter mechanism. *Curr. Opin. Struct. Biol*. 18, 726–33.

41. Mourez, M., Hofnung, M. and Dassa, E. (**1997**). Subunit interactions in ABC transporters: a conserved sequence in hydrophobic membrane proteins of periplasmic permeases defines an important site of interaction with the ATPase subunits. *EMBO J*. 16, 3066–77.

42. Loo, T.W. and Clarke, D.M. (**1993**). Functional consequences of phenylalanine mutations in the predicted transmembrane domain of P-glycoprotein. *J. Biol. Chem*. 268, 19965–72.

43. Loo, T. W. and Clarke, D. M. (**1995**). Rapid purification of human P-glycoprotein mutants expressed transiently in HEK 293 cells by nickel-chelate chromatography and characterization of their drug-stimulated ATPase activities. *J. Biol. Chem*. 270, 21449–52.

44. 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. *J. Biol. Chem.* 277, 21111-21114.

45. 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, 528–36

46. Dawson, R.J. and Locher, K.P. (**2006**). Structure of a bacterial multidrug ABC transporter. *Nature.* 443(7108), 180-5.

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. *Proc Natl Acad Sci USA.* 104(48), 19005-10

48. Ambudkar, S.V., Kim, I.-W. and Booth-Genthe, C. (**2008**). Relationship between drugs and functional activity of various mammalian P-glycoproteins (ABCB1). *Mini Rev. Med. Chem.* 8, 193–200.

49. Higgins, C.F. (**1992**). ABC Transporters: from microorganisms to man. *Annu. Rev. Cell. Biol*. 8, 67-113.

50. 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 Lett*. 580(4), 1049–55.

51. Saraste, M., Sibbald, P.R., and Wittinghofer, A. (**1990**). The P-loop--a common motif in ATP- and GTP-binding proteins. *Trends Biochem. Sci.* 15(11), 430–4.

52. Loo, T.W. and Clarke, D.M. (**1999**). The Transmembrane Domains of the Human Multidrug Resistance P-glycoprotein Are Sufficient to Mediate Drug Binding and Trafficking to the Cell Surface. J. Biol. Chem. 274(35), 24759–65.

53. Jones, P.M., and George, A.M. (**1999**) Subunit interactions in ABC transporters: towards a functional architecture. *FEMS Microbiol. Lett*. 179, 187-202.

54. Hung, L.W., Wang, I.X.Y., Nikaido, K., Liu, P.Q., Ames, G.F.L., and Kim, S.H. (**1998**) Crystal structure of the ATP-binding subunit of an ABC transporter. *Nature.* 396, 703-7.

55. Smith, P.C., Karpowich, N., Millen, L., Moody, J.E., Rosen, J., Thomas, P.J., and Hunt, J.F. (**2002**) ATP Binding to the Motor Domain from an ABC Transporter Drives Formation of a Nucleotide Sandwich Dimer. *Mol. Cell.* 10, 139-149.

56. Zaitseva, J., Jenewein, S., Wiedenmann, A., Benabdelhak, H., Holland, I.B., and Schmitt, L. (**2005**) Functional characterization and ATP-induced dimerization of the isolated ABC-domain of the haemolysin B transporter. *Biochemistry.* 44, 9680-90.

57. Sauna, Z.E., Nandigama, K., and Ambudkar, S.V. (**2006**) Exploiting reaction intermediates of the ATPase reaction to elucidate the mechanism of transport by P-glycoprotein (ABCB1). *J. Biol. Chem.* 281, 26501-11.

58. van der Does, C., Presenti, C., Schulze, K., Dinkelaker, S., and Tampe, R. (**2006**) Kinetics of the ATP hydrolysis cycle of the nucleotide-binding domain of Mdl1 studied by a novel site-specific labeling technique. *J. Biol. Chem.* 281, 5694-5701.

59. Sauna, Z.E., Muller, M., Peng, X.H., and Ambudkar, S.V. (**2002**) Importance of the conserved Walker B glutamate residues, 556 and 1201, for the completion of the catalytic cycle of ATP hydrolysis by human P-glycoprotein (ABCB1). *Biochemistry.* 41, 13989-14000.

60. Tombline, G., Bartholomew, L.A., Urbatsch, I. L., and Senior, A.E. (**2004**) Combined mutation of catalytic glutamate residues in the two nucleotide binding domains of P-glycoprotein generates a conformation that binds ATP and ADP tightly. *J. Biol. Chem.* 279, 31212-20.

61. Tombline, G., Muharemagic, A., White, L.B., and Senior, A. E. (**2005**) Involvement of the "occluded nucleotide conformation" of P-glycoprotein in the catalytic pathway. *Biochemistry* 44, 12879-86.

62. Tombline, G., and Senior, A.E. (**2005**) The occluded nucleotide conformation of P-glycoprotein. *J. Bioenerg. Biomembr.* 37, 497-500.

63. Janas, E., Hofacker, M., Chen, M., Gompf, S., van der Does, C., and Tampe, R. (**2003**) The ATP Hydrolysis Cycle of the Nucleotide-binding Domain of the Mitochondrial ATP-binding Cassette Transporter Mdl1p. *J. Biol. Chem.* 278, 26862-69.

64. Sauna, Z.E., and Ambudkar, S.V. (**2007**) About a switch: how P-glycoprotein (ABCB1) harnesses the energy of ATP binding and hydrolysis to do mechanical work. *Mol. Cancer Ther.* 6, 13-23.

65. Senior, A.E., Al-Shawi, M.K., and Urbatsch, I.L. (**1995**) The catalytic cycle of P-glycoprotein. *FEBS Lett.* 377, 285-89.

66. Urbatsch, I.L., Sankaran, B., Weber, J., and Senior, A.E. (**1995**). P-glycoprotein is stably inhibited by vanadate-induced trapping of nucleotide at a single catalytic site. *J Biol Chem*. 270(33), 19383-90.

67. Urbatsch, I.L., Sankaran, B., Bhagat, S., and Senior, A.E. (**1995**). Both P-glycoprotein nucleotide-binding sites are catalytically active. *J Biol Chem.* 270(45), 26956-61.

68. Sauna, Z.E., Kim, I.W., Nandigama, K., Kopp, S., Chiba, P., and Ambudkar, S.V. (**2007**). Catalytic cycle of ATP hydrolysis by P-glycoprotein: evidence for formation of the E.S reaction intermediate with ATP-gamma-S, a nonhydrolyzable analogue of ATP. *Biochemistry*. 46(48), 13787-99.

69. Martin, C., Berridge, G., Mistry, P., Higgins, C., Charlton, P., and Callaghan, R. (**2000**) Drug binding sites on P-glycoprotein are altered by ATP binding prior to nucleotide hydrolysis. *Biochemistry* 39, 11901-6.

70. 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, 4312-4322.

71. Lu, G., Westbrooks, J.M., Davidson, A.L., and Chen, J. (**2005**) ATP hydrolysis is required to reset the ATP-binding cassette dimer into the resting-state conformation. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17969-74.

72. 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. *Br. J. Pharmacol*. 122, 765-71.

73. Pascaud, C., Garrigos, M., and Orlowski, S. (**1998**) Multidrug resistance transporter P-glycoprotein has distinct but interacting binding sites for cytotoxic drugs and reversing agents. *Biochem. J.* 333, 351-358.

74. Orlowski, S., Mir, L.M., Belehradek, J., and Garrigos, M. (**1996**). Effects of steroids and verapamil on P-glycoprotein ATPase activity: progesterone, desoxycorticosterone, corticosterone and verapamil are mutually non-exclusive modulators. *Biochem. J.* 317, 515-522.

75. Liu, R., and Sharom, F.J. (**1996**). Site-directed fluorescence labeling of P-glycoprotein on cysteine residues in the nucleotide binding domains. *Biochemistry* 35, 11865-73.

76. Romsicki, Y., and Sharom, F.J. (**1999**) The membrane lipid environment modulates drug interactions with the P-glycoprotein multidrug transporter. *Biochemistry* 38, 6887-6896.

77. 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. *J Biol Chem*. 277(44), 41303-6.

78. 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. *J Biol Chem*. 278(3), 1575-8.

79. Loo, T.W., Bartlett, M.C., Detty, M.R., and Clarke, D.M. (**2012**). The ATPase activity of the P-glycoprotein drug pump is highly activated when the N-terminal and central regions of the nucleotide-binding domains are linked closely together. *J Biol. Chem*. 287(32), 26806-16.

80. 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. *J. Biol. Chem.* 276(18), 14972-79.

81. Loo, T.W. and Clarke, D.M. (**2002**). Location of the Rhodamine-binding Site in the Human Multidrug Resistance P-glycoprotein. *J. Biol. Chem*. 277, 44332-38.

82. Loo, T.W. and Clarke, D.M. (**2000**). The Packing of the Transmembrane Segments of Human Multidrug Resistance P-glycoprotein Is Revealed by Disulfide Cross-linking Analysis. *J. Biol. Chem*. 275, 5253-56.

83. 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. *J. Biol. Chem*. 276, 36877-80.

84. Loo, T.W. and Clarke, D.M. (**1997**). Drug-stimulated ATPase activity of human P-glycoprotein requires movement between transmembrane segments 6 and 12. *J Biol Chem*. 272(34), 20986-9.

85. Lomize, M.A., Lomize, A.L., Pogozheva, I.D. and Mosberg, H. I. (**2006**). OPM: orientations of proteins in membranes database. *Bioinformatics*. 22, 623–25.

86. Hrycyna, C.A., Airan, L.E., Germann, U.A., Ambudkar, S.V, Pastan, I., Gottesman, M.M. (**1998**). Structural flexibility of the linker region of human P-glycoprotein permits ATP hydrolysis and drug transport. *Biochemistry*. 37, 13660–73.

87. Sato, T., Kodan, A., Kimura, Y., Ueda, K., Nakatsu, T., Kato, H. (**2009**). Functional role of the linker region in purified human P-glycoprotein. *FEBS J.* 276, 3504–16.

88. Ferreira, R.J., dos Santos, D.J., Ferreira, M.-J., Guedes, R.C. (**2011**). Toward a better pharmacophore description of P-glycoprotein modulators, based on macrocyclic diterpenes from Euphorbia species. *J. Chem. Inf. Model* . 51, 1315–1324.

89. Wiese, M. and Pajeva, I.K. (**2001**). Structure-activity relationships of multidrug resistance reversers. *Curr. Med. Chem*. 8, 685–713.

90. Pajeva, I.K. and Wiese, M. (**2009**). Structure-activity relationships of tariquidar analogs as multidrug resistance modulators. *AAPS J*. 11, 435–44.

91. Ekins, S., Kim, R.B., Leake, B.F., Dantzig, A.H., Schuetz, E.G., Lan, L.-B., Yasuda, K., Shepard, R.L., Winter, M.A., Schuetz, J.D., Wikel, J.H. and Wrighton, S.A. (**2002**). Three-Dimensional Quantitative Structure-Activity Relationships of Inhibitors of P-Glycoprotein. *Mol. Pharmacol*. 61, 964–73.

92. Shapiro, A.B. and Ling, V. (**1997**) Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. *Eur. J. Biochem*. 250, 130–37.

93. Shapiro, A. B. and Ling, V. (**1998**). Transport of LDS-751 from the cytoplasmic leaflet of the plasma membrane by the rhodamine-123-selective site of P-glycoprotein. *Eur. J. Biochem*. 254, 181–88.

94. Parveen, Z., Stockner, T., Bentele, C., Pferschy, S., Kraupp, M., Freissmuth, M., Ecker, G.F., Chiba, P. (**2011**). Molecular dissection of dual pseudosymmetric solute translocation pathways in human P-glycoprotein. *Mol. Pharmacol*. 79, 443–52.

95. Shapiro, A.B., Fox, K., Lam, P., Ling, V. (**2001**). Stimulation of P-glycoprotein-mediated drug transport by prazosin and progesterone. *Eur. J. Biochem*. 2001, 259, 841–50.

96. Wang, E., Casciano, C. N., Clement, R.P. and Johnson, W.W. (**2001**). The farnesyl protein transferase inhibitor SCH66336 is a potent inhibitor of MDR1 product P-glycoprotein. *Cancer Res*. 61, 7525–29.

97. Ekins, S., Kim, R.B., Leake, B.F., Dantzig, A.H., Schuetz, E.G., Lan, L.-B., Yasuda, K., Shepard, R.L., Winter, M.A., Schuetz, J.D., Wikel, J.H. and Wrighton, S.A. (**2002**). Application of three-dimensional quantitative structure-activity relationships of P-glycoprotein inhibitors and substrates. *Mol. Pharmacol*. 61, 974–81.

98. Melchior, D.L., Sharom, F.J., Evers, R., Wright, G.E., Chu, J. W.K., Wright, S.E., Chu, X. and Yabut, J. (**2012**). Determining P-glycoprotein drug interactions: evaluation of reconstituted P-glycoprotein in a liposomal system and LLC-MDR1 polarized cell monolayers. *J. Pharmacol. Toxicol. Methods*. 65, 64–74.

99. Storch, C.H., Theile, D., Lindenmaier, H., Haefeli, W.E. and Weiss, J. (**2007**). Comparison of the inhibitory activity of anti-HIV drugs on Pglycoprotein. *Biochem. Pharmacol*. 73, 1573–81.