# Molecular Modeling Studies of ABC Transporters Involved in Multidrug Resistance

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**Abstract:** Multidrug resistance (MDR) is a limitation to cancer chemotherapy, antibiotic treatment and HIV medication. Molecular models of the ABC transporters ABCB1 (P-glycoprotein), ABCC4 (multidrug resistance protein 4 (MRP4)) and ABCC5 (MRP5), which are involved in MDR, may aid in the development of drugs inhibiting anticancer agents efflux.

**Key Words:** Molecular modeling, P-glycoprotein/ABCB1, MRP4/ABCC4, MRP5/ABCC5, multidrug resistance.

## INTRODUCTION

A particular limitation to treatment of diseases related to the major causes of mortality globally, e.g. cancer chemotherapy and antibiotic treatment, is development of multidrug resistance. Cells exposed to toxic compounds can develop resistance by a number of mechanisms, including increased excretion. Excretion of ions and small organic molecules, which are often too hydrophilic to penetrate the cellular membrane on their own, requires a transport protein.

In general, transporter proteins have a recognition site making them specific for a particular substrate, and drugs may exert their effect by binding to transporters and either inhibiting transport of the substrate or functioning as a false substrate for the transport process (Fig. **1**). In order to overcome multidrug resistance problems, development of inhibitors of drug efflux transporters has been sought for use as a supplement to current therapy [1].

The information on transporter structure and function is rapidly increasing, and the focus on transporters as drug targets is growing. In the future, more drugs interacting with transporters probably will be developed.

## ATP-BINDING CASSETTE (ABC) TRANSPORTERS

The ATP-binding cassette (ABC) transporters represent an important class of targets for discovery of drugs for treatment of a broad range of human diseases. These transporters belong to Class 3 (Primary active transporters) in the transporter classification approved by the transporter nomenclature panel of the International Union of Biochemistry and Molecular Biology [2]. Active transport uses the free energy stored in the high-energy phosphate bonds of adenosine triphosphate (ATP) as energy source to activate the transporter. The ABC transporters comprise a family of structurally related membrane proteins that share a common intracellular structural motif in the domain that binds and hydrolyses ATP, and they use the energy from ATP directly by exhibiting ATPase activity to cleave ATP's terminal phosphate, moving substances from regions of low concentration to regions of high concentration.

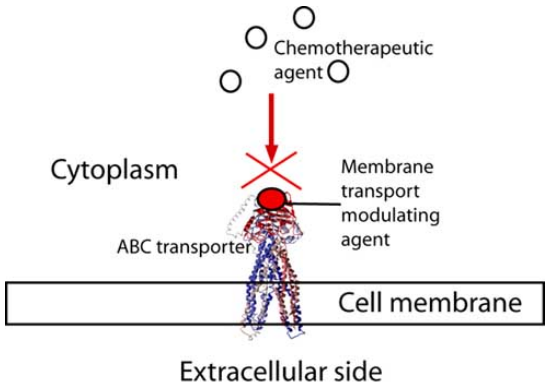


**Fig. (1).** Membrane transport modulating agent inhibiting drug efflux of chemotherapeutic agent from cancer cell by binding to ABC transporter. ABC (ATP-Binding-Casette) transporters belong to a large family of ATP-dependent transporters, for which the hydrolysis of ATP by an ATPase force the substrate through the membrane, often against a concentration gradient. The ABC transporter displayed is a molecular model of ABCC4 [40].

ABC transporters have both trans-membrane domains (TMDs) and intracellular nucleotide binding domains (NBDs), and the domain arrangement of these transporters is generally TMD-NBD-TMD-NBD (Fig. **2**). However, domain arrangements such as TMD-TMD-NBD-TMD-NBD, NBD-TMD-NBD-TMD, TMD-NBD and NBD-TMD also occur [3, 4]. The NBD contains the Walker A and B motifs [5] and a signature C motif, and the substrate specificity is provided by the TMDs, which contain 6–11 transmembrane helices (TMHs) [4, 6].

The human genome encodes more than 40 ABC transporters divided into five different subfamilies based on phylogenetic analysis: ABCA, ABCB, ABCC, ABCD and ABCG. Subfamilies ABCE and ABCF are related to ABC transporters, but they lack transmembrane domains and thus

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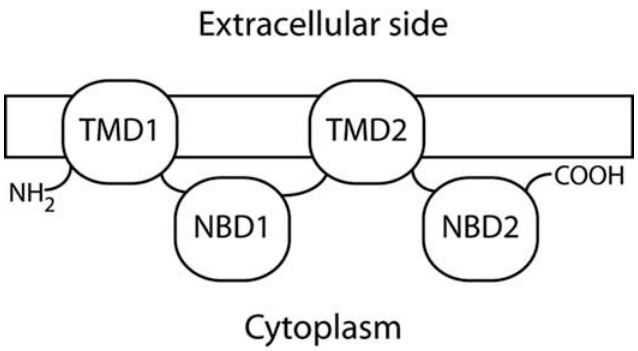


**Fig. (2).** General TMD-NBD-TMD-NBD domain arrangement of ABC transporters such as ABCB1, ABCC4 and ABCC5.

are not membrane transporters [4, 6]. Since ABC genes are highly conserved between species, most of these genes have probably been present since the beginning of eukaryotic evolution [4]. Transporters in subfamilies ABCA, ABCB, ABCC and ABCG are involved in multidrug resistance [7-10].

### ABCB1 (P-Glycoprotein)

ABCB1 (P-glycoprotein) actively pumps chemotherapeutic agents, such as adriamycin, vincristine and daunorubicin, out of cancer cells, resulting in multidrug resistance to such drugs. ABCB1 has broad substrate specificity and may have evolved as a defense mechanism against toxic substances. ABCB1 is widely distributed in normal cells in tissues such as the mammary gland, prostate gland, salivary gland, sweat glands of the skin, pancreatic ducts, renal tubules, and in bile canaliculi and ductules, the gastrointestinal epithelium, epithelia of the bronchi, in adrenal and in endothelial cells at blood–brain barrier sites and other blood–tissue barrier sites [11]. The highest ABCB1 expression is observed in tumors from colon, adrenal, pancreatic, mammary and renal tissue, even in the absence of prior chemotherapy [12]. In breast cancer, neuroblastoma, various types of leukemia, and several sarcomas, negative prognostic implications of ABCB1 expression have been established [12]. Still, the relationship between ABCB1 expression and response to chemotherapy remains unclear.

### ABCC4 (MRP4)

ABCC4 exports organic anions, including endogenous and exogenous substances, and is involved in multidrug resistance [13-17]. ABCC4 is expressed in brain [18], kidney [19], liver [20], erythrocytes [21], platelets [22], adrenal gland [23], and pancreas [24]. According to experimental studies, ABCC4 is involved in resistance to the anticancer agent topotecan, thus protecting the brain from chemotherapy. This suggests that the therapeutic efficacy of central nervous system-directed drugs that are ABCC4 substrates may be improved by developing ABCC4 membrane transport modulating agents [14].

### ABCC5 (MRP5)

ABCC5 transports cGMP out of cells and is also involved in multidrug resistance [25]. It is expressed in most tissues, such as in vascular smooth muscle cells, cardiomyocytes, and vascular endothelial cells in the heart [26], in placenta [27], in human erythrocytes [25], in skeletal muscle, kidney, testis, heart and brain [28-30], in smooth muscle cells of the corpus cavernosum, ureter and bladder, and mucosa in ureter and urethra [16, 31].

## MOLECULAR MODELING

The binding of drugs to targets in the body is highly structure- and stereospecific, implying that only drugs with certain chemical groups and spatial orientation has high affinity to a certain drug target. Molecular modeling can be used to investigate the molecular interactions between drugs and drug targets, aiding the search to understand the intermolecular forces involved in determining the potency and the specificity of the drug. Insight into structural changes of the drug and the drug target for adopting an energetically favorable complex can aid to predict how a designed drug will fit into the drug target. In theory, new chemical compounds with fewer side effects may eventually be designed to act as drugs.

### Homology Modeling of Drug Targets

Experimental 3-dimensional structures of proteins from X-ray crystallography have increased relevant structural information for making molecular models of drug targets. However, most drug targets are membrane proteins, and making crystals of membrane proteins is technically difficult. A molecular model of a human drug target with unknown structure may be constructed by homology modeling using a protein with a known 3D crystal structure with a sequence similarity, so called homology, to the drug target as a template. Homology between two proteins having a common ancestor is determined by sequence similarity, indicating the presence of similar features such as homologous protein fold. Still, since their amino acid composition in the binding site area may differ from each other, two homologous proteins may bind different drugs. In general, the 3D structure of homologous proteins is more conserved than sequence, and a protein structure can provide a close general model for other proteins if the sequence identity is greater that 50%.

The main steps of homology modeling are template identification, target-template alignment, model building, model refinements, and validation of model.

### Template Identification

Template identification involves matching the protein structure of interest (target) to experimentally determined structures. In order to construct a molecular model by homology, at least one protein (template) assumed to have the same 3D structure as the target is required [32].

### Target-Template Alignment

After template identification, an optimal target-template alignment must be made, identifying corresponding positions in the target and the template. Ideally, the predicted structure of the target, based on the template, will be as similar as possible to an experimental structure of target. It is recommended to use a multiple sequence alignment as a basis for the target-template alignment, since it highlights evolution-

ary relationships and increases probability that corresponding sequence positions are correctly aligned [32].

### Model Building

Model building in general involves construction of the core areas of the model, based on homology to the template, and construction of loops. An example of core modeling is construction of the model from a few core sections defined by the average of Cα atoms in the conserved regions of the alignment [32].

### Model Refinements

After model building, the model can be refined using molecular mechanics software. In molecular modeling, both molecular mechanics and quantum mechanics calculations may be used. Quantum mechanics is used for calculations concerning electronic system, while for calculations concerning atomic nuclei, molecular mechanics is used. The basis for using classical mechanics calculations comes from the Born-Oppenheimer approximation, stating that atomic nuclei move much slower than electrons, so the vibrational and rotational motions of a molecule can be separated from the electronic motion [33]. In molecular mechanics the atomic structure of a molecule is considered to be a collection of atomic masses that interact with each other *via* harmonic forces, and molecular mechanics calculations are performed by equations based on Newton's Classical Mechanics [34]. Examples of molecular mechanics calculations are energy minimization, molecular dynamics calculations [35], and Monte Carlo simulations [36, 37]. The Laws of Thermodynamics state that molecules seek the lowest potential energy spontaneously, and energy minimization is the calculation of the lowest energy conformation of a molecule. Molecular dynamics is the simulation of molecular motion during a short period of time. Monte Carlo simulations may sample conformational space of a molecule by random moves followed by a local energy minimization, and the complete energy is then calculated. The resulting molecular conformation is accepted or rejected based on the energy and the temperature [36].

Geometries and structures of small molecules can be predicted by calculation of the energy of the electronic system using quantum mechanics, but protein molecules are too big to be solved by the Schrödinger equation ($H\psi=E\psi$). Quantum mechanics calculations can be used for calculation of electrostatic potentials (ESP) of small molecules.

### Model Validation

Molecular models should in general be considered as working tools for generating hypotheses and designing further experimental studies related to protein structure and function, and drug interactions. Ideally, an iterating process towards a better understanding of protein structure and function of these proteins is contributed by site-directed mutagenesis studies and molecular modeling. Docking of drug molecules into their putative binding site and identifying amino acids in the protein model interacting with the drug molecule will aid the selection of amino acids for further site-directed mutagenesis studies. One may consider the model as partly correct if the observations of drug binding affinities made in the experimental observations are in accordance with the effects proposed by the modeling study. If not, an adjustment of the model must be performed, thus experimental studies based on assumptions made from the models may be useful for further model refinements. In the light of philosophy of science, molecular modeling may follow the key idea of falsificationism, a philosophy of science introduced by Karl Popper that has the key idea that scientific theories are falsifiable [38]. According to Popper, science starts with problems, and, as solution to the problem, scientists propose falsifiable hypotheses. Science progresses by trial and error when hypotheses are tested. From a falsificationistic point of view, the scientific problem to be tested is the 3 dimensional protein structure. A molecular model of the protein is constructed as a hypothesis. Drug molecules are docked into the putative binding site of the model, and logical predictions are deducted from the hypothesis by suggesting amino acids that may be involved drug binding. The hypothesis is tested experimentally by making single point mutations in the protein and testing drug binding affinities.

## MODELING STUDIES OF HUMAN ABC TRANSPORTERS

### Templates

Choosing the right template is essential in homology modeling. The multidrug transporter *Staphylococcus aureus* Sav1866 was determined by X-ray crystallography at 3.0Å resolution in an outward-facing conformation in 2006 [39]. Sav1866 belongs to the ABC superfamily, and it has a ~23-31% sequence identity to human ABCB1, ABCC4 and ABCC5 [40, 41]. Even though accurate predictions of protein structure may require an amino acid sequence similarity greater than 50 % between the target and the template protein, there may be considerable structural similarities with lower homologies as well. The classic example is the structural similarity between the G-protein coupled receptors and bacteriorhodopsin, where the sequence similarities within the transmembrane regions are 6–11% [42]. Also relevant is the conservation of the secondary structure elements, such as the TMDs and the NBDs of the ABC transporters, since active sites and functional domains can have very similar geometries, even for distantly related proteins. Phylogenetic analyses of ABC transporters have indicated that eukaryotic ABCB transporters (including ABCB1), ABCC transporters (including ABCC4 and ABCC5), and bacterial ABC transporters have a common ancestor, and that they have similar domain organizations [43]. Mutagenesis studies have confirmed that there is a structural relationship between Sav1866 and ABCB1 [44], and among the ABCC transporters, ABCC4 and ABCC5 are most similar structurally to ABCB1, indicating that the Sav1866 X-ray crystal structure can be used as a template for constructing ABCC4 and ABCC5 models by homology. In contrast to ABCC1, ABCC2 and ABCC3, which contain a TMD0 domain in their N-terminal end, ABCC4 and ABCC5 lacks the TMD0 domain, giving ABCC4 and ABCC5 ABCB1-like core structures [45]. A recent study combining small angle X-ray scattering data and cryo-electron crystallography data of ABCB1 and comparing the structures with the Sav1866 X-ray crystal structure [39] has demonstrated that modeling of eukaryotic transporters on the basis of bacterial counterparts may yield

realistic models [46]. In our ABC transporter modeling studies, the Sav1866 X-ray crystal structure [39] apparently was the only suitable template.

The Sav1866 consists of two subunits, each with a transmembrane domain–nuclear binding domain (TMD-NBD) topology, with six TMHs in each TMD. In ABCB1, ABCC4 and ABCC5, the genes have fused into a monomer with a TMD-NDB-TMD-NBD topology. In the Sav1866 X-ray crystal structure [39], the two subunits are twisted and embracing each other. Both the TMDs and NBDs are tightly interacting, and bundles of TMHs diverge into two "wings" towards the extracellular side. Each wing consists of TMH1 and TMH2 from one subunit and TMH3–TMH6 from the other subunit.

Four x-ray structures of the bacterial ABC lipid flippase, MsbA, was published in 2007 [47], trapped in different conformations, two nucleotide-bound structures and two in the absence of nucleotide. However, the resolution of the data (3.7Å for one of the structures and only Cα carbons for the rest) was not good enough for construction homology models of ABCB1, ABCC4 and ABCC5. Three MsbA structures published in 2001 [48], 2003 [49] and 2005 [50] where retracted in 2006 [51], since it was realized that the biological interpretations based on the MsbA structures were invalid.

**Molecular Models of ABCB1, ABCC4 and ABCC5**

Since the 3 dimensional structures of ABCB1, ABCC4 and ABCC5 have not been experimentally determined, we have used molecular modeling by homology based on the Sav1866 X-ray crystal structure [39] to gain structural insight into their potential as drug targets [40, 41, 52]. Fig. (**3**) displays the ABCB1 model [41], and the predicted pdb coordinates of the ABCB1, ABCC4 and ABCC5 models can be downloaded from http://www2.uit.no/www/ansatte/organisasjon/hjem//artikkel?p_document_id=85698&p_dimension _id=30477.

The electrostatic potential surface calculated from molecular models may be used to study substrate difference between different drug targets. ABCB1 transports cationic amphiphilic and lipophilic substrates [53-56], while ABCC5 transports organic anions [25, 57], and according to molecular modeling studies [41, 52], the electrostatic potential surface of the substrate translocation area of the ABCB1 model is neutral with negative and weakly positive areas, while the electrostatic potential surface of the ABCC5 model substrate translocation chamber generally is positive. Thus, ABCB1, transporting cationic amphiphilic and lipophilic substrates, has a more neutral substrate translocation chamber than ABCC5, which has a positive chamber transporting organic anions [41].

Since site-directed mutagenesis studies can be used as a validity test of molecular modeling studies, such data on a putative verapamil binding site in ABCB1 may serve as an illustrative example for validation of the ABCB1 model [41]. A verapamil binding site in ABCB1 including residues Leu65 (TMH1) [58], Ile306 (TMH5) [58], Ile340 (TMH6) [58, 59] and Phe343 (TMH6) [60] has been suggested from site directed mutagenesis studies. In a molecular model of ABCB1, these residues may form a binding site [41], indicating that the Sav1866 X-ray structure [39] may serve as a suitable template for ABCB1 modeling. The corresponding residues in ABCC4 and ABCC5 shown in Table **1** and Fig. (**4**) are possible candidates for single point mutations. Fig. (**5**) displays the alignment of the TMHs 1, 5, and 6 among the three transporters. As described in our previous ABCB1 modeling study [41], cross-linking studies on ABCB1 indicating that TMH6 and TMH12 take part in ligand binding [58-61], that TMH5 and TMH 8 are near each other [62], and that TMH2 and TMH11 are near each other [63], also confirms the validity of the ABCB1 model.

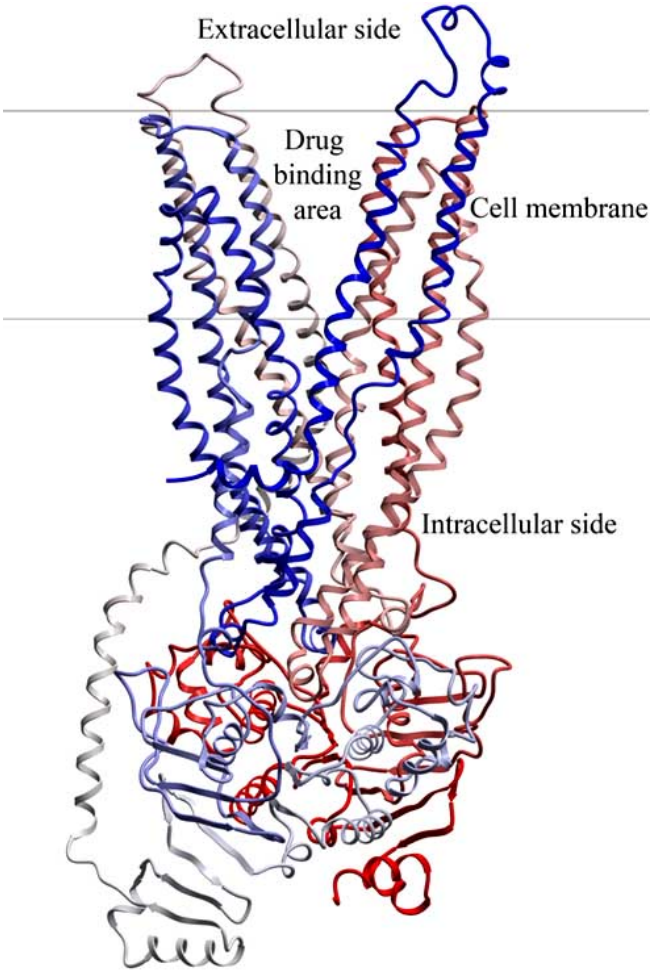


**Fig. (3).** Backbone Cα-trace of ABCB1 model viewed in the membrane plane. Color coding: blue *via* white to red from N-terminal to C-terminal.

Multidrug transporters transport a broad range of structurally diverse molecules, and this promiscuous feature contributes to special challenges in trying to map the substrate-binding site in multidrug transporters. A cysteine-scanning mutagenesis and oxidative cross-linking study of substrate-induced changes in ABCB1 has shown that the packing of the TMHs surrounding the drug-binding site changes when ABCB1 binds to a particular substrate [64], and this induced-fit mechanism may explain how ABCB1 can transport a

**Table 1. Residues Suggested from Site Directed Mutagenesis Studies to Take Part in a Verapamil Binding Site, and the Corresponding Residues in ABCC4 and ABCC5**

| TMH | ABCB1 (Site Directed Mutagenesis Data) | ABCC4 | ABCC5 |
|---|---|---|---|
| 1 | Leu65 [58] | Glu103 | Gln190 |
| 5 | Ile306 [58] | Ser328 | Val410 |
| 6 | Ile340 [58, 59] | Gly359 | Asn441 |
| 6 | Phe343 [60] | Arg362 | Thr444 |

broad range of compounds, including stereoisomers of substrates. Furthermore, as it has been demonstrated from structural analyses of the bacterial multidrug binding protein QacR, which binds a broad spectrum of structurally dissimilar cationic, lipophilic drugs, there is little effect on substrate binding affinity when substrate binding residues identified from crystal structure studies are removed. This may be explained by the presence in the pocket of a redundancy of polar, charged, and aromatic residues that are capable of electrostatic neutralization [65].

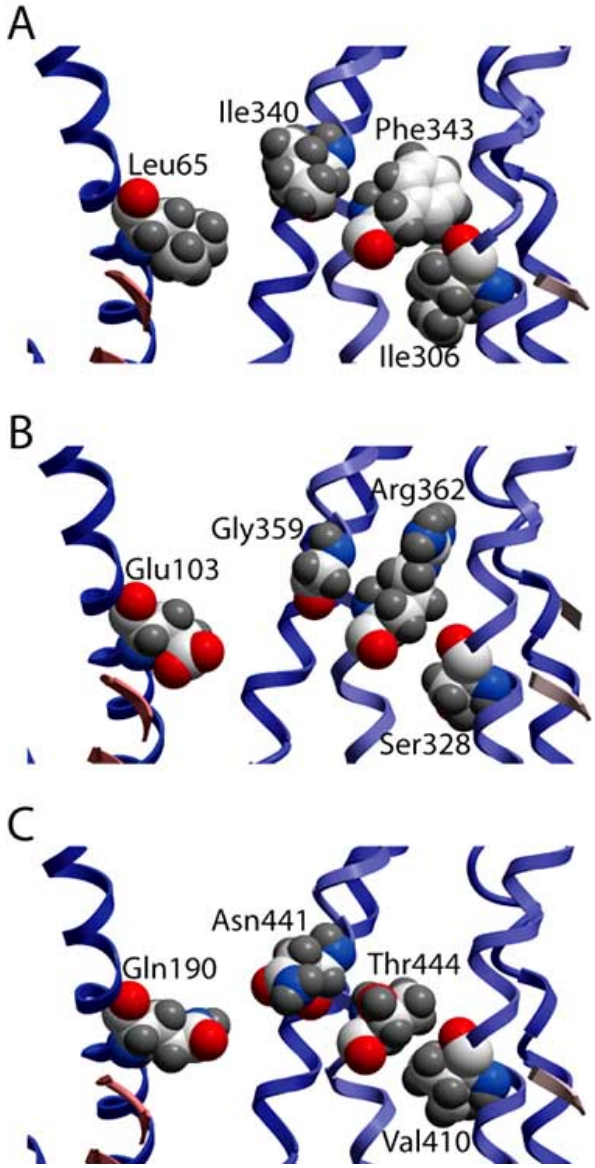


**Fig. (4).** Close-up cross sections of putative drug binding sites of ABCB1 (**A**), ABCC4 (**B**) and ABCC5 (**C**) transporter models viewed in membrane plane (cytoplasm downwards). Residues displayed are suggested from site directed mutagenesis studies to take part in a verapamil binding site in ABCB1 (A), and the corresponding residues in ABCC4 (**B**) and ABCC5 (**C**).

## TRANSPORT MECHANISM OF ACTION OF ABC TRANSPORTERS

The conformation of the crystal structure of Sav1866 indicates that ABC transporters may use an "alternating access and release" mechanism for transport where ATP binding and hydrolysis control the conversion of one state into the other, and that subunit twisting and domain swapping takes place in the transport cycle [39]. Experimental studies have shown that multiple allosteric substrate binding sites may be present in the ABC transporter TMDs [17], thus, substrates bind to high affinity binding sites accessible to the intracellular side, and during the translocation process the binding sites change conformation, and the substrates are released to the extracellular side from low affinity binding sites. The ABC transporter models [40, 41, 52] are assumed to be in a conformation representing the substrate releasing conformation. The verapamil binding amino acids listed in Table **1** may interact with substrate during substrate translocation, both in the substrate binding inward facing conformation and the substrate releasing outward facing conformation, since site directed mutagenesis studies indicate that these amino acids participate in a drug binding site [58-60] and the ABC transporter models indicate that these amino acids participate in a substrate releasing site [41].

## DEVELOPMENT OF INHIBITORS OF ABCB1, ABCC4 AND ABCC5

Development of inhibitors of ABCB1, ABCC4 and ABCC5 may help to prevent efflux of anticancer agents. Such inhibitors are not cytotoxic agents themselves, but when used in combination with cancer drugs which are normally pumped out by the cell by these transporters, intracellular drug concentrations are maintained, restoring sensitivity to these therapeutics.

During the last 30 years, three generations of ABCB1 inhibitors have been developed [1, 12]. The first generation of ABCB1 inhibitors were established clinically used compounds that were discovered to also function as ABCB1 inhibitors, largely by chance [12]. These compounds were not

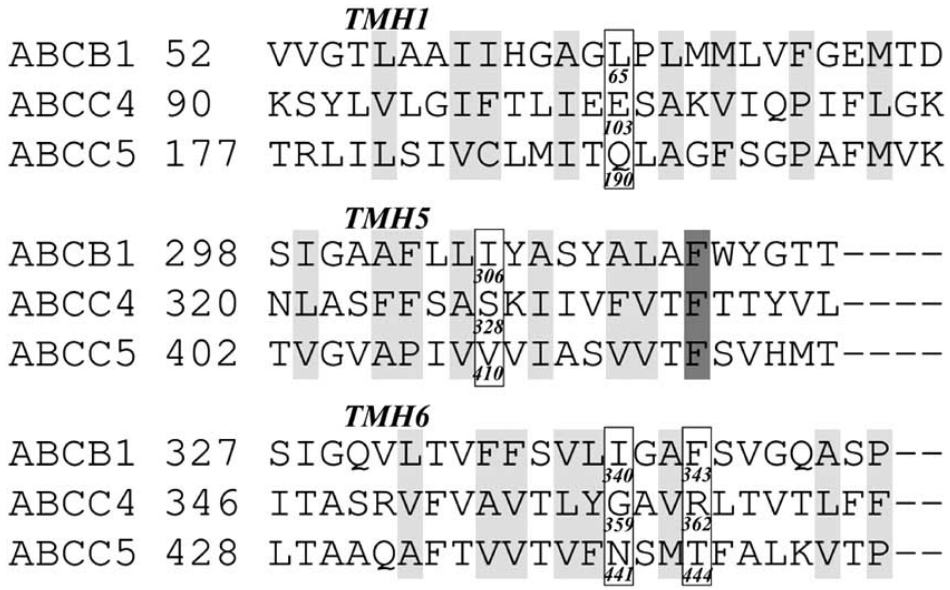


**Fig. (5).** Alignment of TMHs 1, 5, and 6 among ABCB1, ABCC4 and ABCC5. Boxed residues are suggested from site directed mutagenesis studies to take part in a verapamil binding site in ABCB1, and the corresponding residues in ABCC4 and ABCC5.

selective, produced undesirable side effects, and were in general less potent than later generations of ABCB1 inhibitors [1]. The second-generation ABCB1 inhibitors were based on the structures of the first-generation compounds and optimized using Quantitative structure-activity relationship (QSAR) [12]. Although second-generation ABCB1 inhibitors were less toxic, dangerously high doses of both first- and second-generation ABCB1 inhibitors were needed, and they also exhibited toxicity due to an increased availability of the co-administered chemotherapy [1].

While the first- and second generations of ABCB1 inhibitors failed to demonstrate the desired clinical benefit, third-generation ABCB1 inhibitors, discovered by combinatorial chemistry screening [12], are more potent and more selective than earlier compounds, [12, 66]. Four promising third-generation lead compounds have been developed by combinatorial chemical screens (Fig. **6**), Elacridar (Pharmaceutical code name GF120918) [67], Zosuquidar (Pharmaceutical code name LY335979) [68], Tariquidar (Pharmaceutical code name XR9576) [69], and Ontogen (Pharmaceutical code name OC144-093) [70]. These compounds have the ability to modulate ABCB1 function at the nanomolar concentration range and are currently in clinical trials [12].

However, the therapeutic benefit of ABCB1 inhibition is yet to be firmly established. Continued development of ABCB1 inhibitors may establish the true therapeutic potential of ABCB1-mediated multidrug resistance reversal. Ideally, a balance between the positive effects of ABCB1 inhibition at the tumor site and the negative potential toxic side effects outcome of reducing elimination of the chemotherapy can be achieved. Even though molecular modeling is an important tool that can help to design ABCB1 inhibitors, it should be noted that while a potential drug may be promising *in silico* and *in vitro*, it may be toxic *in vivo*, as has been demonstrated with the first generation ABCB1 inhibitor verapamil [12].

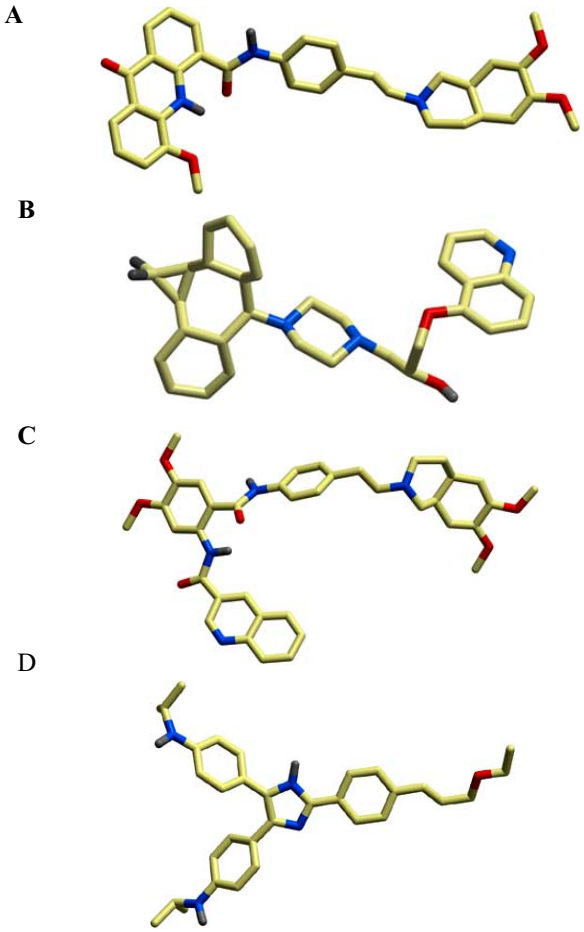


**Fig. (6).** Chemical structures of third-generation lead compounds developed by combinatorial chemical screens; A. Elacridar, B. Zosuquidar, C. Tariquidar, and D. Ontogen. Color coding: Blue: nitrogen; red: oxygen; grey: hydrogen; dark grey: fluorine.

From a structure aided drug design point of view, the specificity and affinity of ABC transporter substrate binding

is of particular interest. The ABCB1, ABCC4 and ABCC5 models [40, 41, 52] that are based on the Sav1866 X-ray crystal structure [39] are in an outward facing, substrate releasing conformation, but a conformation open to the intracellular side is more representative for the high affinity substrate binding and may be more suitable for investigating high affinity substrate recognition. Still, information about the molecular properties of the substrate translocation pathway of ABCB1, ABCC4 and ABCC5 can be used to design therapeutic agents that may aid to reduce the consequences of multidrug resistance.

Coordinates of the ABCB1, ABCC4 and ABCC5 models are available from the authors upon request, and on the website: http://www2.uit.no/www/ansatte/organisasjon/hjem//artikkel?p_document_id=85698&p_dimension _id=30477.

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