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Computational models for prediction of interactions with ABC-transporters

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The polyspecific ligand recognition pattern of ATB-binding cassette (ABC)-transporters, combined with the limited knowledge on the molecular basis of their multispecificity, makes it difficult to apply traditional molecular modelling and quantitative structure–activity relationships (QSAR) methods for identification of new ligands. Recent advances relied mainly on pharmacophore modelling and machine learning methods. Structure-based design studies suffer from the lack of available protein structures at atomic resolution. The recently published protein homology models of P-glycoprotein structure, based on the high-resolution structure of the bacterial ABC-transporter of Sav1866, may open a new chapter for structure-based studies. Last, but not least, molecular dynamics simulations have already proved their high potential for structure–function modelling of ABC-transporter. Because of the recognition of several ABC-transporters as antitargets, algorithms for predicting substrate properties are of increasing interest.

Introduction

Transmembrane transporters are indispensably involved in the absorption, tissue distribution, excretion and toxicity, as well as pharmacokinetics and pharmacodynamics, of drugs. Members of the multidrug ATB-binding cassette (ABC) transporter subfamily have attracted particular interest, since they, in addition to their physiological role in tissue protection, actively extrude a large variety of therapeutically administered drugs from malignant cells and, thus, are responsible for multiple drug resistance in cancer patients [1]. Inhibition of the most intensively studied transporters, ABCB1, ABCC1 and ABCG2, has been advocated as a mechanism for the restoration of drug sensitivity [2]. Additionally, there is increasing evidence that cholestatic forms of drug-induced liver damage result from a drug- or metabolite-mediated inhibition of hepatobiliary transporter systems, such as ABCB1, ABCB4, ABCG2, ABCG5 and ABCG8 [3]. Therefore, interaction with ABC-transporters determines, to a large extent, the clinical usefulness, side effects and toxicity risks of drugs. Thus, detailed three-dimensional information on the molecular basis of drug-transporter interaction would have large potential value in assisting rational design of new drugs and establishing *in silico* models for the prediction of absorption; distribution; metabolism; elimination; toxicity (ADMET) and safety problems. Most of the clinically relevant ABC-transporters, however, show a rather fuzzy and promiscuous pattern of ligand specificity. ABCB1, ABCC1 and ABCG2, the three key transporter involved in multiple drug resistance in tumour therapy, efflux a broad panel of structurally and functionally diverse compounds, which range from low molecular weight compounds such as cyclosporines, up to lipids [4]. This inherent promiscuity of ABC-transporters, accompanied by the limited knowledge on the molecular basis of this multispecificity renders traditional molecular modelling methods rather ineffective for generation of global predictive models. Nevertheless, there have been considerable modelling efforts to target promiscuous proteins, especially in the field of cytochromes [5] and the human ether-a-go-go-related gene (hERG) potassium channel [6], and the ABC-transporter field definitely can benefit from the experiences gained with these (anti)targets.

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Ligand-based design studies

P-glycoprotein

In view of the lack of high-resolution structures, inhibitor design has been advanced by ligand-based approaches. In lead optimization programs, numerous quantitative structure–activity relationships (QSAR) studies on structurally homologous series of compounds have been performed. Within the past decade, verapamil analogues, triazines, acridonecarboxamides, phenothiazines, thioxanthenes, flavones, dihydropyridines, propafenones and cyclosporine derivatives have been extensively studied, and the results are summarized in several reviews [7,8]. These studies indicate that, primarily, global physicochemical parameters (lipophilicity, molar refractivity), H-bond acceptor strength and the special arrangement of H-bond acceptors correlate with P-glycoprotein inhibitory activity. Systematic and quantitative structure-activity relationship studies have mainly been performed in the groups of phenothiazines and propafenones [9]. For the latter Hansch- and Free-Wilson analyses, hologram QSAR (HQSAR), comparative molecular field analysis (CoMFA) and comparative molecular similarity index analysis (CoMSIA) studies, as well as non-linear methods and similarity-based approaches, have been pursued [10]. Hansch-analyses typically give a picture showing excellent correlation between lipophilicity and pIC_{50} values within structurally homologous series of compounds [11]. This further supports the hypothesis that the interaction with the protein takes place within the membrane bilayer. Thus, lipophilicity of the compounds might trigger their concentration at the binding site rather than influencing binding itself. However, 3D-QSAR studies, based on CoMFA and CoMSIA, revealed favourable hydrophobic interactions along the propanolamine chain and in the vicinity of the phenyl-ring of the arylpiperazine moiety [12], which favours a more space directed influence of lipophilicity. This space-directed character of lipophilicity was also demonstrated by Pajeva and Wiese for a series of phenothiazines and thioxanthenes [13] and a subset of our propafenone-based library [14].

Taking all the information from QSAR studies together, the consensus picture remains rather general. Strong inhibitors are characterized by high lipophilicity (and/or molar refractivity) and possess at least two H-bond acceptors. Other features, such as H-bond donors and π–π-stacking, may act as additional interaction points. Furthermore, some steric constraints seem to apply in the vicinity of pharmacophoric structures. These basic requirements are further supported by various pharmacophore modelling studies performed by Pajeva and Wiese [15], Garrigues *et al.* [16], Ekins *et al.* [17], Chang *et al.* [18] and our own group [19]. Interestingly, all pharmacophore models retrieved are highly predictive for new ligands, but show clear differences, both in the number and type of features involved, and in the special arrangement of these features. This further strengthens the special characteristics of the ABCB1-binding site/region/zone.

In order to obtain global models, and taking into account the *non-lock and key* like binding principle, several authors applied non-linear methods, such as support vector machines and artificial neural networks, for prediction of ABCB1 ligands. Kaiser *et al.* projected a set of 131 propafenone-type P-glycoprotein inhibitors onto a self-organizing map, using a set 2D-autocorrelation vectors. Showing a good separation between actives and inactives, the size of the map was enlarged and the propafenones were merged with the SPECS compound library (134,000 compounds). After repeating the training procedure, those SPECS compounds which localized close to the most active propafenones were retrieved and tested for pharmacological activity [20]. All seven hits obtained in this virtual screening event showed completely different scaffolds from the propafenones. Pharmacological testing revealed that six compounds had IC_{50} values <10 μM and two compounds showed inhibitory activity with IC_{50} values in the sub-micromolar range, which definitely renders them new lead compounds for ABCB1 (Figure 1).

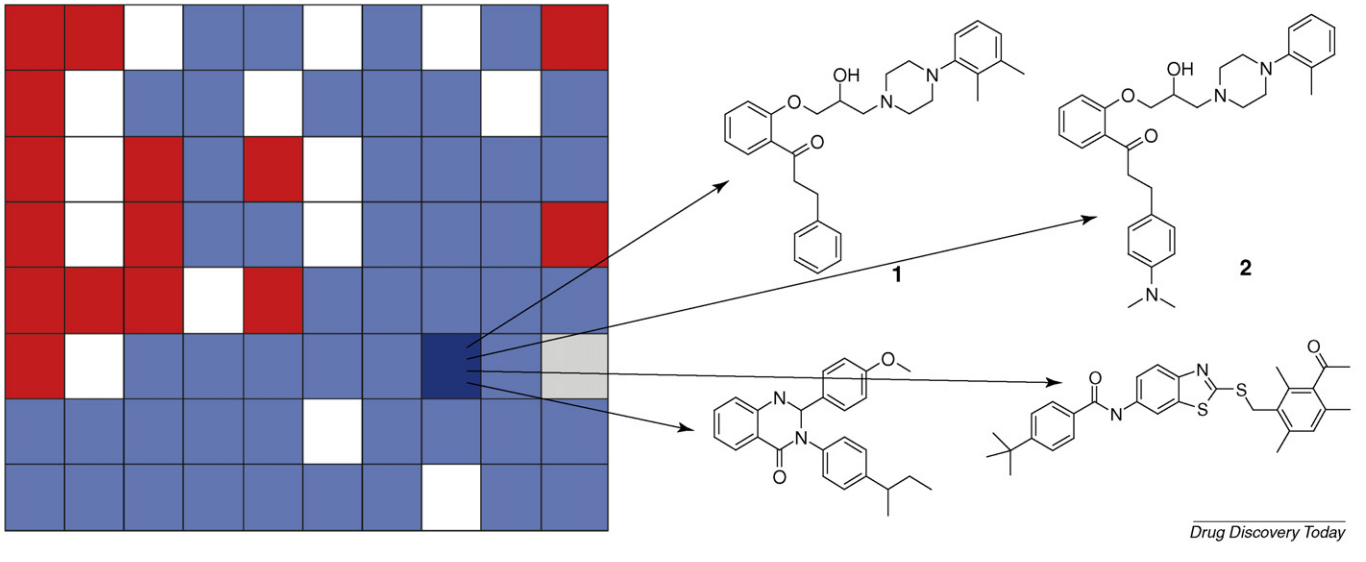

FIGURE 1

Hit compounds from the SPECS compound library co-localising in the same neuron as highly active propafenone-type inhibitors of P-glycoprotein. Taken from Kaiser *et al.* [20].

Other ABC-transporters

Within the last decade, inhibitors of the multidrug resistance (MDR)-related proteins ABCC1 (MRP1) and ABCC2 (MRP2), the breast cancer resistance protein ABCG2 (BCRP) and the sister of P-glycoprotein ABCB11 (SPGP, bile salt export pump (BSEP)) have been published [21].

Analogously to ABCB1, a lot of structurally and functionally diverse inhibitors for ABCC1 have been identified [22]. These include verapamil, flavonoids, raloxifene, isoxazoles, quinazolinones, quinolines, pyrrolopyrimidines and peptides. For the flavonoids, three structural characteristics seem to be of major importance: the total number of methoxy-groups, the number of OH-groups and the dihedral angle between ring B and ring C.

Analogous to ABCB1 and ABCC1, ABCG2 also has a broad, partly overlapping and diverse substrate specificity, transporting mitoxantrone, methotrexate, camptothecins (topotecan, irinotecan), anthracyclines, etoposide, and flavonoids [23]. The latter have also served as lead structures for the development of ABCG2 inhibitors. Zhang *et al*. selected a panel of 25 flavonoids, covering five different structural subclasses, in order to identify structural features important for ABCG2 inhibitory activity. Also, the highly selective natural product fumitremorgin served as a starting point for synthesis of a series of 42 structural analogous indolyl diketopiperazines [24]. Within a series of propafenone analogues, lipophilicity was shown to be highly predictive for ABCG2 inhibitory potency. QSAR studies, using a set of 10 global descriptors (e.g. lipophilicity, polar surface area, number of H-bond donors and acceptors, number of rotable bonds), revealed that hydrophobicity, number of rotable bonds and number of H-bond acceptors are key features both for activity and selectivity towards ABCB1 [25]. Results further indicate that, for the class of propafenones, ABCG2 is more tolerant for structural modification than ABCB1. Selectivity is, therefore, mainly determined by the distinct QSAR pattern with respect to ABCB1, rather than a specific interaction with ABCG2 (Figure 2).

Other ABC-proteins capable of transporting drugs comprise ABCC3 (MRP3), ABCC4 (MRP4), ABCC5 (MRP5) and ABCA2 [2]. These proteins are of increasing interest in various disease areas and the above-mentioned computational methods might also be applied to these transporters both for identification of inhibitors and for selectivity profiling. However, currently only few *in vitro* data are available for these transporters and QSAR studies with adequate validation sets are, therefore, rather rare.

Prediction of substrates – the anti-target concept

Within recent years and also in light of the fact that all inhibitors of ABCB1 entering clinical studies so far have failed, prediction of substrate properties of compounds has gained a lot of interest in the Pharmaceutical Industry. ABC-transporters are involved in the uptake, distribution and elimination of drugs and play, therefore, a vital role in determining the bioavailability of candidate compounds. Especially in the case of CNS-active drugs, which have to cross the blood–brain barrier, antihistamines (which should not cross the blood–brain barrier) and compounds with low aqueous solubility, their substrate properties are intrinsically connected to their clinical applicability. In contrast to inhibitors, however, accurate data sets for ABC-transporter substrates are rare and only approximately 300 compounds are available in the literature. Computational methods applied span the whole range of classification algorithms, utilizing decision trees [26], discriminant analysis [27], self-organising maps [28] and support vector machines [29]. Seelig proposed a general recognition pattern for ABCB1 substrates, based on a set of structural elements related to H-bond acceptor characteristics [30]. In analogy to Lipinski's rule of five, Didziapetris *et al*. used a set of 220 compounds to introduce the 'rule of fours': compounds where the number of N and O atoms $\geq$8, molecular weight >400 and acid pK_a < 4 are likely to be ABCB1 substrates, whereas compounds with the number of N and O atoms $\leq$4, molecular weight <400 and base pK_a < 8 are likely to be non-substrates [31]. Gombar and Polli derived thresholds for molecular E-states [27], Cabrera *et al*. pursued a topological substructural approach [32], and de Cerqueira *et al*. utilized combinatorial QSAR [33]. Models obtained generally show a total accuracy of around 80%. In this field the success of computational models will heavily rely on the availability of large, accurate data sets taking into account the fact that some compounds described as inhibitors are also substrates, such as, for example verapamil and cyclosporine. Thus, one has carefully to decide whether the model should predict the 'macroscopic' picture (i.e. net transport beyond the membrane) or the 'microscopic' one (substrate property on the molecular level). The respective data sets will have to be derived from different experimental protocols [34] and the outcome always will only reflect the quality of the input data.

Structure-based approaches

The successful application of structure-based drug design has been demonstrated for several targets, including tyrosine kinases and proteases, leading to (among others) the development of potent anticancer agents [35]. Structure-based design relies on the availability of structures of the target protein at the atomic level. Integral membrane proteins are notoriously resistant to forming diffracting crystals and available structures are, therefore, rare with only 144 unique resolved structures to date [36]. P-glycoprotein was the first multidrug transporter for which low-to-medium resolution data were obtained, whereas a high-resolution structure is still not available. Structure-based design in this case has to resort to protein homology modelling, based on inference of structural homology between a structurally resolved protein and a protein of unknown structure. Selection of templates is based on sequence homology and an identical number of predicted transmembrane spanning helices.

Publication of the first structure of a full length ABC-transporter, the lipid A transporter MsbA from *E. coli* at 4.5 Å in 2001 [37], and the subsequent appearance of the transporter from *V. cholerae* at 3.8 Å in 2003 [38] seemed to open a path for protein homology modelling of ABCB1. Though the nucleotide binding domain (NBD) structures were irreconcilable with that of structurally-resolved NBDs of other ABC-transporters (16 at present; summarized in [39]) and with results from NBD/NBD cysteine cross linking data [40], the positioning of helices within individual transmembrane domains (TMDs) was similar in both the *E.coli* and the *V. cholerae* structures. Also, the helix pairs 5/8 and 2/11, which formed the contact interfaces between the two TMDs in the *V. cholerae* MsbA structure, were found to be in close proximity in ABCB1 as indicated by cross-linking with copper phenanthroline. This led to two assumptions: *first*, that MsbA might represent a

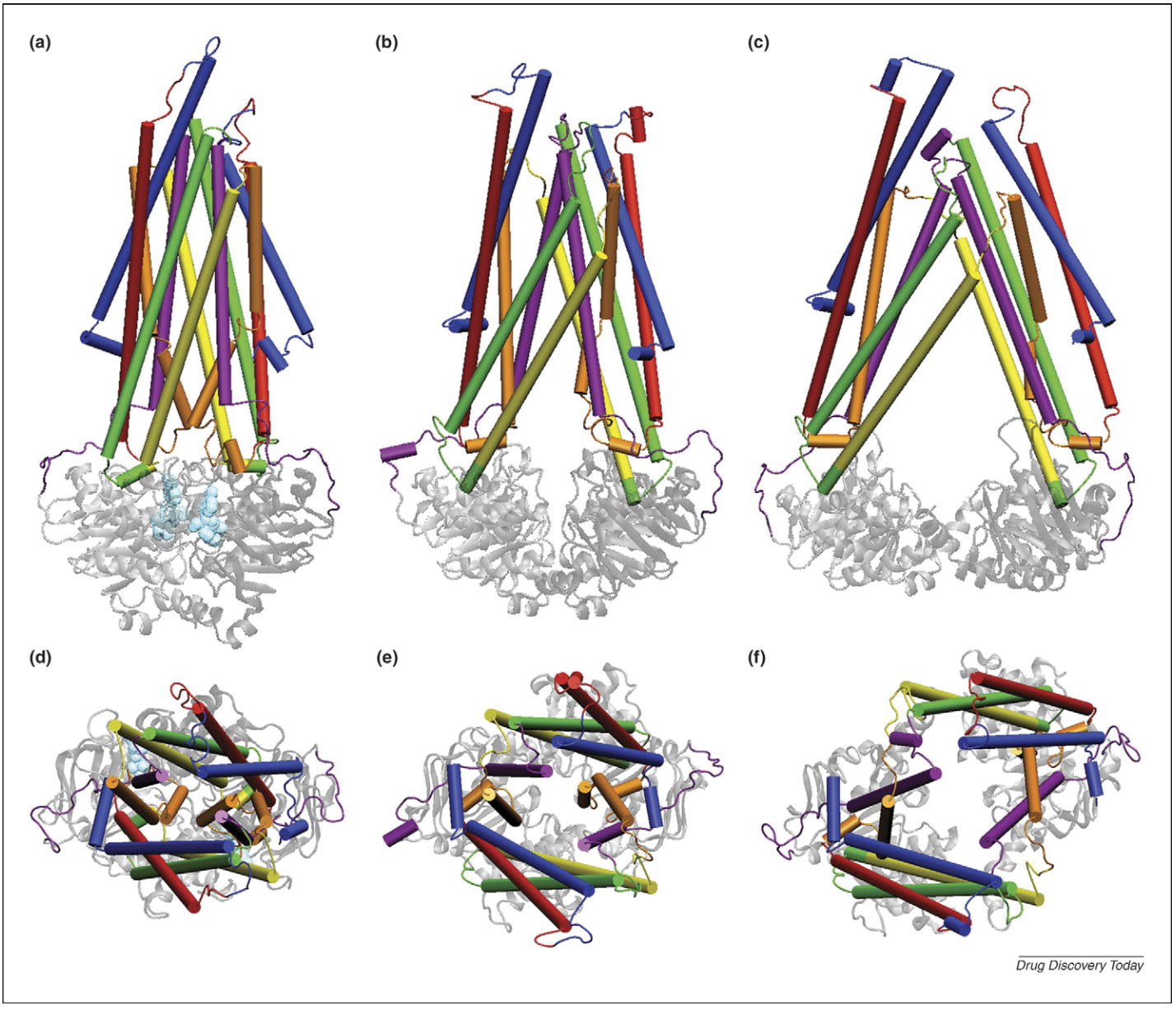

FIGURE 2

Closed, semi-open and open state protein homology model of P-glycoprotein based on the high resolution structure of the bacterial transporter Sav1866. Taken from O'Mara and Tielemann [50].

good template structure for ABCB1 and *second*, that the experimental evidence for ABCB1 was reconcilable with the structure of MsbA and, thus, supported the validity of this structure. Both assumptions turned out to be wrong.

In September 2006 the first structure of a putative multidrug transporter from methicillin resistant *Staphylococcus aureus* (MRSA) was published [41]. This structure, which was resolved to 3.0 Å, contained two bound ADP molecules sandwiched between the NBDs. The overall RMSD between the ADP-bound and the subsequently published AMP-PNP bound structure [42] was low (0.097 Å for 1116 of 1156 visible residues) suggesting that both structures represent the ATP-bound state of the NBDs. Sav1866 is functional as a homodimer of two identical polypeptide chains, each consisting of an N-terminal TMD fused to a C-terminal NBD, while in ABCB1 all four domains are fused into a single polypeptide chain. The NBDs of the Sav1866 structures (PDB codes: 2HYD and 2ONJ) show a canonical head-to-tail orientation with two bound nucleotides. The overall architecture differs from the side-by-side arrangement of TMDs found for the ABC-importers BtuCD [43], Hl1470/1 [44], $ModB_2C_2$ [45], and the maltose transporter MalFGK2 [46] in that the two TMDs were twisted. Each TMD thereby forms the majority of contacts with the opposite and not the ipsilateral NBD. Experimental evidence from well-controlled TMD/NBD cross-linking experiments seems to indicate that this is also the case for ABCB1 [47]. These data and a sequence homology of Sav1866 with each, the N- and C-terminal half of ABCB1 of 28% now encourage its use as a template structure for the generation of protein homology models. Superpositioning of the MsbA structures and the novel Sav1866 structures indicated a possible problem with the handedness of the MsbA structures

and also raised concerns about their suggested topology. Consequentially, all three MsbA structures were retracted along with two structures of a bacterial multidrug transporter, EmrE in December 2006 [48]. In this context it has to be noted that several authors used the wrong structures for generation of protein homology models and as starting point for molecular dynamics simulations. The results of these studies have to be carefully reconsidered, as some of them simply might be wrong. In a recent publication by the group of Chang, the three previously published structures from *Escherichia coli*, *Vibrio cholerae* and *Salmonella typhimurium* were revised and compared to a closed-conformation AMP-PNP bound structure of *S. typhimurium* MsbA resolved to 3.7 Å [49]. The latter structure shows that MsbA and Sav1866 have essentially identical architecture. Structure prediction of P-gp can now be pursued both using the Sav1866 high-resolution full-length structures, as very recently shown by O'Mara and Tielemann [50], and using the corrected structures provided by Ward *et al*. However, the problem of the low sequence similarity in the transmembrane domains remains and protein homology models have been taken very cautiously when using them as starting points for structure-based design attempts.

Homology models are comparable to medium resolution structures and normally not of sufficient quality to be used for structure based design directly. Nevertheless, a biochemical data based improvement of these models is possible by molecular dynamics simulations. Molecular dynamics simulations are a powerful tool to study function and dynamics of macromolecules at an atomic level of details. These methods are routinely applied to water soluble proteins and several force fields are available. Simulations of proteins embedded in membranes are more challenging for several reasons: (i) the difficulty to construct an appropriate starting structure with the protein embedded in an equilibrated membrane; and (ii) the high viscosity and, therefore, slow motion and long equilibration times of the membrane environment. The biological membrane environment is a complex mixture of molecules containing among others different types and concentrations of lipid molecules, cholesterol and embedded proteins. The complexity of the membrane environment is reduced in simulations of embedded proteins and a membrane is typically represented by a water hydrated phospholipid bilayer containing monovalent ions.

Campbell *et al*. [51] created a full atom MsbA model based on the first published MsbA structure that included only Ca atoms. The model was further optimized by molecular dynamics simulations in the membrane mimicking octane slap. The dimer model resulted unstable in the TMD region. Haubertin *et al*. [52] studied the transmembrane domain of MsbA in the open state structure and found a quite stable semipore-like structure. Vandevuer *et al*. [53] created a model of ABCB1 based on the MsbA structure and used rigid body molecular dynamics simulations to further improve their model. Experimentally derived cross-linking data were taken into account as distance restraints to guide the structural optimization. Unfortunately, as explained in the structure model section, these efforts were in vain, because of the retraction of the MsbA template structure. In the near future new studies based on the Sav1866 structure of *Staphylococcus aureus* are very likely to appear.

Several simulations of ABC transporters have appeared in the literature that were based on the retracted MsbA structures. Vandevuer *et al*. [53] investigated the open structure of MsbA in an octane slap as a membrane mimic. While the NBD were stable, their simulations pointed towards problems in the TMD region, which turned out to be unstable. Haubertin *et al*. [52] studied the same open structure of MsbA in a phospholipid bilayer. The authors found significant intra and inter-helical changes, but did not identify the same structural instability. Sonne *et al*. [55] predicted the structure of ABCB1 using experimental restraints to drive the MD based conformational search starting from the closed MsbA structures, resulting in large changes in the TMD region. These studies indicate that molecular dynamics simulations, if carried out carefully, can be a valuable tool to test models for their consistency and to point towards potential structural problems.

Several studies have appeared in recent years on BtuCD, an ABC transporter consisting of 20 transmembrane helices, with a putative mode of function that differs from ABCB1 or MsbA. In an early simulation study Oloo and Tieleman [54] showed that ATP binding induces conformational changes in the NBD, bringing the two nucleotide binding domains into closer contact. This movement was propagated to the membrane spanning domain where it stimulates conformational rearrangements. The coupling between the NBDs, the TMDs and the conformational changes triggered by ATP binding have been studied by Sonne *et al*. [55], using perturbed elastic network calculations and biased molecular dynamics simulations. These calculations indicate that ATP binding affects the NBDs dimer and a transition in their relative arrangement is coupled with the TMDs and with the central pore. ATP binding was found to induce the closure of the pore, while the release of ATP triggered gate opening.

An extensive simulation study, comparing the behavior of the BtuD NBD dimer, the BtuCD integral membrane protein complex and the BtuCDF complex, including the periplasmic binding protein BtuF, gave some very intricated insights [56]. The ATP-bound BtuD dimer was found to form a closed symmetric dimer, but the ATP binding to the BtuCD complex did not result in the same symmetrical closed NBD structure. The bacterial ABC importers, such as BtuCD require a periplasmic binding protein to deliver the substrate to the transporter and it is known that the binding of the periplasmic binding protein to the integral membrane transporter stimulates ATP binding and hydrolysis. When the BtuF periplasmic binding protein was added to the system to form the BtuCDF complex, an asymmetric ATP bound state was formed in the NBD. The simulations indicate that the BtuCD complex is conformationally restricted and this constraint of the membrane-bound complex seems to prevent the formation of a symmetric ATP-bound NBD dimer. The authors found that the signal of the binding of BtuF to the importer, carrying the vitamin B_{12} substrate, can reach the NBD via conformational changes, dominate by a rotation of the transmembrane domain BtuC moving in opposite direction of the NDB domains BtuD. This domain rearrangement can promote the formation of an asymmetric tightly ATP bound state. This finding, if confirmed by experiments, does strongly speak in favor of an alternating hydrolysis mechanism.

Nucleotide-binding domain

The nucleotide-binding domain of the ABC transporter provides the energy for the transport of substrates across the membrane.

Molecular dynamics simulations have been used to investigate the structural basis of the conversion of the chemical energy stored in the phosphate bond of the ATP into the mechanical energy and molecular motions of the NBD that is subsequently consumed by the translocation of substrates. Several NBDs have been studied in the apo form or bound to the nucleotides ADP or ATP.

In early studies of the monomeric HisP NBD [57,58] and, very recently MJ0796 [59], the structural effects of nucleotide binding to the NBD were investigated. Those studies observed ATP dependent intradomain motions with the helical subdomain of the NBD monomer moving closer to the bond nucleotide. The presence of Mg^{2+} was found to modulate the effect.

The functionally relevant configuration of the NBD in ABC transporter is a dimer where the nucleotides can bind between two NBD molecules. In recent times, several simulations of dimeric NBDs have appeared in the literature, including MJ0796 [54,60], MalK [61] and BtuD [58]. In the simulations of the dimeric systems the authors report that the binding of ATP induced the formation of tightly bound, symmetric and very stable structures, while removing the nucleotides from the complex typically induced an increase in the NBD–NBD distance and an increase in structural fluctuations. The authors typically highlighted large changes in the relative arrangement and distance of the NBDs, but did not report intradomain motions as found in the earlier studies of the monomeric NBDs. Only a very recent study by Jones and George reported intradomain conformational changes, focusing on structural effects of ATP, ADP and no nucleotide. The helical domain was found to move relative to the core domain, with the most closed structure identified in the presence of ATP. The Gln in the Q-loop was reporter to be crucial in this transition, as it establishes the contact between the helical domain and the γ-phosphate of the ATP. The effect of ATP hydrolysis was approximated by modifying one ATP to ADP. The symmetry of this ATP/ADP system was perturbed as the helical subdomain of one NBD molecule undergoes a large conformational transition, not seen in the ATP/ATP simulations.

Traditional 2D- and 3D-QSAR-methods heavily rely on the basic assumption that all compounds used bind to the same site and in the same mode to the target protein. However, in the case of ABC-transporter there is experimental evidence that drug-binding occurs at the interface of the two transmembrane domains [62] and, therefore, the binding cavity is rather large accommodating simultaneously up to three ligands at least in some of the transporters [63]. Thus, the molecular basis of ligand–transporter interaction still needs to be elucidated. In light of both the lack of atomic detail structures available and the polyspecificty of the transporters, this requires intense combination of biochemical, biophysical, ligand-based and structure-based design methods, as recently demonstrated for the benzodiazepine-binding site of the $GABA_A$-receptor. Although the binding site is located in a subdomain/subdomain interface and includes a highly flexible loop, the combination of cysteine scanning, site directed mutagenesis, photoaffinity labelling and pharmacoinformatics allowed the identification of two distinct binding hypotheses which fulfil all experimental data [64].

In virtual screening attempts, success stories published so far mainly rely on VolSurf/grid independent descriptors (GRIND) descriptors, pharmacophore models, and artificial neural networks. New approaches, such as SVM and similarity-based descriptors, may pave the way for the establishment of predictive *in silico* filters, which could be applied in the early drug discovery phase. This will be of special importance in the field of predicting substrate properties of ABC-transporters, as these are increasingly considered as antitargets in the pharmaceutical industry. However, considering, in addition, the discussion which took place at a recent meeting on *in silico* ADMET [65], it might be more promising to establish a series of local models rather than a global one. Global models might rather be suitable for a rough clustering of large compound libraries into substrates/non substrates. Respective local models will then allow to consider the effects of minor chemical changes within closely related compound series.

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