# Chapter 30
# Multidrug Efflux Pumps and Their Inhibitors Characterized by Computational Modeling

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**Abstract** Antimicrobial resistance is a key public health concern of our era due to an ever-increasing number of drug-resistant pathogens, including several Gram-negative bacilli. The latter are endowed with a low permeable outer membrane and with numerous chromosomally encoded multidrug efflux pumps, which are not only ubiquitous but also polyspecific, thus recognizing a broad range of compounds. Efflux pumps are a major defense mechanism of these organisms against antimicrobials as they can significantly increase the levels of resistance by allowing time for the organisms to develop specific resistance mechanisms. One of the potential strategies to reinvigorate the efficacy of antimicrobials is by joint administration with efflux pump inhibitors, which either block the substrate binding and/or hinder any of the transport-dependent steps of the pumps. In this chapter, we provide an overview of multidrug resistance efflux pumps, their inhibition strategies, and the important findings from the various computational simulation studies reported to date with respect to the rational design of inhibitors and on deciphering their mechanism of action.

**Keywords** Antimicrobial resistance • Efflux pump • ABC • MATE • RND • P-glycoprotein • Efflux pump inhibitor • Molecular dynamics • Molecular docking

## 30.1 Introduction

Decades ago, when the incidents of bacterial resistance were not widespread and newer antimicrobial agents were continually being discovered, it was not surprising to hear that the era of infectious diseases caused by microbes was virtually over [1]. However, over the last two decades, there has been a dramatic surge in the number

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of multidrug-resistant bacteria, yet paradoxically the number of pharmaceutical companies developing new antimicrobial drugs has dwindled. These coincidences have collectively made antimicrobial resistance one of the world's most demanding health problems [2, 3].

With continuous efforts to develop better antimicrobial agents against such resistant microbes, successful milestones are being reached in the case of infections caused by Gram-positive organisms [4], while the Gram-negative pathogens (e.g., *Enterobacteriaceae*, *Acinetobacter*, and *Pseudomonas*) still prove to be a major challenge due to their very high intrinsic drug resistance. This intrinsic resistance is largely attributed to the permeability barrier imposed by the outer membrane (OM) and to the expression of chromosomally encoded drug efflux pumps [5].

Drug efflux pumps are ubiquitously expressed protein complexes residing in the membrane to expel a wide range of structurally diverse antimicrobials and toxins, thereby lowering their concentration inside the cell to sub-toxic levels [6–9]. They also enjoy a special status of being considered a part of the primary survival kit of microorganisms as these polyspecific pumps remove most of the xenobiotics from the cell interior to give the organism time to acquire resistance to agents through more specific adaptive mechanisms [10, 11]. This way the efflux mechanisms likely contribute to a rapid emergence of resistance in the presence of antimicrobial selection pressure. Efflux mechanism also interplays with other resistance mechanisms to significantly increase the levels and profiles of resistance [12].

The current shortage of new antimicrobials in the development pipeline to replace the ineffective ones adds to the urgency to protect the efficacy of existing drugs. One possible way of reinvigorating the previously effective drugs attenuated by bacterial efflux mechanism is by the combinatorial use of efflux pump inhibitors (EPIs). This chapter provides an overview of the various multidrug resistance (MDR) efflux pumps with particular emphasis on computational studies of their inhibitors that have been reported to date. After a brief introduction on the importance of efflux pumps for MDR, we describe the major families of multidrug transporters, their mechanism of function, and the various inhibition strategies. Moreover, we summarize the molecular modeling studies that facilitate our progress in developing efficacious inhibitors for better management of efflux-mediated MDR. For any family of MDR pumps, we focus here mostly on studies including the data from molecular dynamics (MD) simulations when they are present (either in addition to docking or exploiting experimental information about structures of receptor-inhibitor complexes). The reader is referred to the relevant literature on studies making exclusive use of molecular docking and other computational methods that are cheaper compared to MD simulations [13–42].

## 30.2 Efflux-Mediated Resistance and MDR

Bacteria have evolved a multitude of mechanisms that in solitude or in combination with each other function to counter the effectiveness of drugs and overcome the deleterious effect of any antimicrobial agents, thus making the bacteria

resistant to multiple drugs. These mechanisms [43–45] include (i) the alteration of the macromolecular drug target either through chemical modification or by mutation to insensitive variants (e.g., alteration of penicillin-binding protein in methicillin-resistant *Staphylococcus aureus* [46]); (ii) the protection of the target via the production of immunity proteins, alteration of metabolic pathways (e.g., elimination of the requirement of *para*-aminobenzoic acid in sulfonamide-resistant bacteria for the synthesis of folic acid and nucleic acids); (iii) the direct chemical modification or inactivation of the antibiotics (e.g., enzymatic inactivation of β-lactams by β-lactamases); (iv) the altered transport of the compounds into the cell (e.g., reduced membrane permeability barrier with decrease in production of porins); and (v) the increased active efflux of drugs out of the cell through efflux pumps.

Among the aforementioned resistance mechanisms, the efflux-mediated approach, where pumps actively export substrate molecules from the cytoplasm to the external medium in an energy-dependent manner, is the predominant one in MDR [47], working in synergy with the low permeability of the OM in Gram-negative bacteria to keep a tight check on the entry of unwanted toxic compounds. Indeed, drug molecules that have gained access to the periplasmic space can further penetrate the cytoplasmic (inner) membrane via diffusion, but they can be expelled out of the cell either by single-component pumps (e.g., Tet pumps [48]) or by multicomponent pumps (e.g., AcrAB-TolC of *Escherichia coli* and MexAB-OprM of *Pseudomonas aeruginosa* [47, 49, 50]).

The wide distribution and overlapping functions of MDR efflux pumps in bacteria hint at their probable role in physiological functions in addition to mediating intrinsic and acquired MDR [51]. A few of these functions include virulence, stress response, bacterial cell communication, colonization, fitness and intracellular survival, and transport of toxic compounds (as in the case of MacAB-TolC which is involved in exporting an extracellular peptide enterotoxin produced by enterotoxigenic *E. coli*) [52].

MDR pumps also function as either a preexisting mechanism or an activated resource in response to numerous cellular stresses caused by antibiotics and other chemical substances such as bile salts, fatty acids, and ethanol that are often substrates of pumps relevant for drug resistance. For instance, AcrAB, the major pump belonging to the resistance-nodulation cell division (RND) superfamily of transporters in enteric bacteria living within the intestinal tract, is upregulated under such stress conditions enabling the bacterial survival in host organisms [43]. The major facilitator superfamily (MFS) pump, MdtM, also functions with AcrAB-TolC in a synergistic manner to protect *E. coli* from bile salt stress [53]. Also, NorM [54], a multidrug and toxic-compound extrusion (MATE) family transporter, and MacAB [55], a macrolide-specific ATP-binding cassette (ABC) superfamily exporter, protect the bacteria against oxidative stress. In the case of *P. aeruginosa*, several Mex pumps are upregulated in response to various stress triggers like membrane-damaging or ribosome-disrupting agents, reactive oxygen species, and/or nitrosative stress [47, 56, 57].

Apart from the previously mentioned functions, MDR pumps have also been identified to play a substantial but varying role in the formation and survival of

biofilms in different species. For instance, the loss or inhibition of any of nine MDR pumps or the TolC OM protein in *Salmonella* impairs its biofilm-forming ability with reduced production of curli [58]. Similarly, *E. coli* mutants with a genetic deletion of one of the MDR pump genes results in reduced biofilm formation [59].

## 30.3 Classification of Drug Efflux Pumps

The transport proteins have been successfully classified by Milton Saier's group in over 800 families on the basis of functional and phylogenetic information (Transporter Classification Database: http://www.tcdb.org) [60]. The transporter genes identified in hundreds of sequenced bacterial genomes have also been documented in Ian Paulsen's database (http://www.membranetransport.org) [61]. Among the numerous families of transporters, the prominent ones responsible for MDR can be divided into two major groups based upon bioenergetical and structural features [51]: (i) Primary active transporters belonging to the ABC superfamily hydrolyze ATP as a source of energy. (ii) Secondary active transporters utilize the proton (or sodium) gradient as a source of energy (the proton motive force is an electrochemical gradient in which the movement of hydrogen ions drives transport of the substrate [62]) and are classified into four superfamilies/families (MFS, MATE, RND, and the small multidrug resistance [SMR]) on the basis of conserved consensus motifs and functional similarities. While the major clinically relevant efflux systems in Gram-positive bacteria are usually non-RND pumps and often the singleton protein pumps belonging to the MFS, MATE, SMR, or ABC, the RND efflux systems are by far the most important in Gram-negative bacteria [12].

## 30.4 Structural and Functional Mechanisms of Drug Efflux Pumps

### *30.4.1 ABC Pumps*

ABC transporters are ubiquitous membrane systems involved in the efflux of toxins, metabolites, and drugs. These transporters are typically composed of two cytoplasmic nucleotide-binding domains (NBDs) and two hydrophobic transmembrane domains (TMDs) [63]. In some transporters, the TMDs, responsible for drug recognition and transport, are fused to highly homologous NBDs, where ATP is hydrolyzed. The NBDs possess the Walker A and B motifs, common to all ATP-binding proteins, and a signature motif, specific to ABC transporters [64]. It has been proposed that ABC efflux pumps were derived from secondary active transporters by superimposition of NBD onto the transporter during evolution [64]. These transporters are found to house multiple drug-binding sites, which is compatible with

their broad substrate specificity and multidrug binding capabilities. Ligand-binding and transport assays have shown that P-glycoprotein (P-gp, ABCB1, MDR1), the most extensively studied ABC member, has at least four pharmacologically distinct binding sites that are allosterically coupled [65, 66]. This family of exporters function with a mechanism termed the ATP switch model [67], where the nucleotide-driven interaction of the NBDs causes reorientation of the TMDs and reduces drug affinity, thereby transporting the substrate (Fig. 30.1a) [70, 71]. Ominous examples of ABC transporters are the mammalian P-glycoprotein active against cytotoxic compounds used in chemotherapy, LmrA of *Lactococcus lactis*, MsbA conferring resistance to erythromycin in Gram-negative bacteria, and MacAB-TolC of *E. coli* able to expel macrolides.

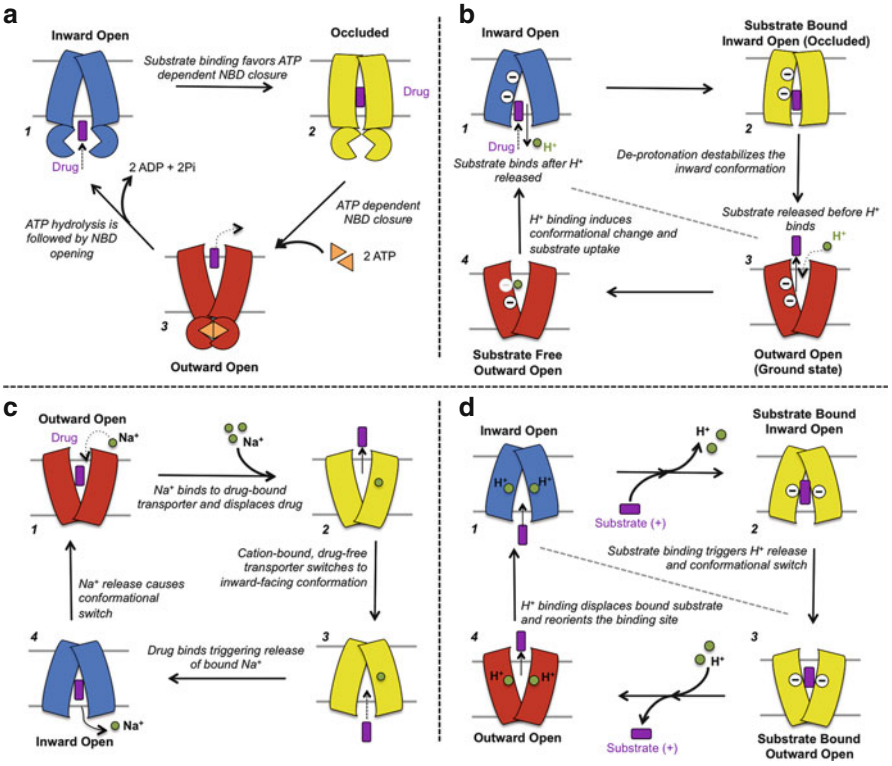



**Fig. 30.1** Transport mechanisms proposed for members of four major families of MDR efflux pumps. (**a**) Simplified drug transport cycle of ABC efflux pumps showing the inward-facing, occluded, and outward-facing states. (**b**) Indirect competition mechanism in MFS multidrug/proton antiporters. (**c**) $Na^+$/multidrug antiport mechanism in transporters of the MATE family. (**d**) Alternating site transport mechanism of EmrE of the SMR family (Transport mechanism of the RND transporters is omitted here but described in detail in Chap. 1 of this book. Obtained with modification and permission from Refs. [63, 68, 69]. (**a**, **b**), are derivatives of figures from Du et al. [63] used under the Creative Commons Attribution License (CC BY). (**c**, **d**) are adapted from Lu et al. [68] and Schuldiner [69], respectively)

### *30.4.2 MFS Pumps*

The MFS pumps belong to the largest group of secondary active membrane transporters [72]. They are omnipresent systems that transport sugars, intermediate metabolites, and drugs and are the major contributors of MDR in Gram-positive bacteria. Most of these pumps are singlet transporters belonging either to 12- or 14-transmembrane segment (TMS) members of the drug/$H^+$ antiporters. In Gram-negative bacteria, they are located in the cytoplasmic membrane and transport drugs from the cytosol to the periplasm from where constitutive RND pumps, such as AcrAB-TolC and MexAB-OprM, may capture and efflux the drug molecules to the external medium, thereby synergistically boosting the activity of these singlet pumps in producing resistance [73, 74]. These transporters operate through an alternating access mechanism (Fig. 30.1b) in which drug-binding sites are alternately exposed to the outside or inside of the cell to uptake and release substrates. Similar to P-glycoprotein, the MFS pumps also contain several distinct (possibly overlapping) allosterically coupled binding sites [75]. There exists an indirect competition between the substrates and protons for binding to their respective different locations, as shown in MdfA of *E. coli*, which might likely play a key role in their transport mechanism [76]. The most studied pumps of this family are NorA of *S. aureus* and its homologs Bmr and Blt of *Bacillus subtilis*, Tet pumps (12-TMS in Gram-negative bacteria and 14-TMS in Gram-positive bacteria) [48], and MdfA [77].

### *30.4.3 MATE Pumps*

Efflux pumps of the MATE family are mainly 12-TMS $Na^+$/drug antiporters that pump substrates from the cytoplasm to the periplasmic space [78]. These transporters are widespread in bacteria and are also found in higher animals and plants. The common substrates of these pumps are cationic dyes, fluoroquinolones, and aminoglycosides. All MATE pump structures show a similar 12-TMS helix topology with an internal twofold sequence similarity reflected in the tertiary structure [68, 79, 80] as N-terminal and C-terminal lobes. These pumps exhibit distinct binding sites for cation and drug enabling their simultaneous binding. The cation binding (with an unusual cation-π interaction with an aromatic ring) and release promote the interconversion between the drug-free and cation-bound configuration and drug-bound configuration as shown in the case of NorM of *Neisseria gonorrhoeae* (Fig. 30.1c) [68].

### *30.4.4 SMR Pumps*

Transporters in the SMR family [81] are the smallest drug efflux proteins known with just 100–120 amino acids folded into four relatively short transmembrane α-helices. They form either a homo- or heterodimer to exchange $H^+$ for pumping out

either monocationic (e.g., ethidium and tetraphenylphosphonium) or dicationic (e.g., methyl viologen) compounds into the periplasm. The orientation of monomeric subunits in the dimer was a long-debated issue with crystallographic data showing antiparallel arrangement of EmrE dimer while chemical cross-linking favoring a parallel arrangement [82]. Although the structure was withdrawn [83], this issue concluded on grounds that it can exhibit a dual topology and that the direction of insertion of the monomeric unit really does not matter for the efflux function [69, 84]. Structural plasticity and flexibility are the basis of multidrug recognition and transport in EmrE, the well-studied pump of this family [85]. This transporter shows functional symmetry where conformational changes in the two monomers result in an interconversion between inward- and outward-facing states [86]. A fixed stoichiometry of two protons is exchanged per substrate molecule, and this results in an electrogenic state for transport of monovalent cations but an electroneutral state for divalent cations [87]. The conserved membrane-embedded glutamate residue (Glu14) in each monomer is essential for proton and substrate binding. Hence, these transporters show an apparently simple, competitive, alternating site mechanism (Fig. 30.1d) in which all substrates bind to the same site [85] and compete with protons for binding [82, 86].

### *30.4.5 RND Pumps*

Efflux pumps of the RND superfamily [88] are the major clinically relevant efflux systems in Gram-negative bacteria also due to their extremely wide substrate specificity [47]. Indeed, some of these transporters are able to recognize hundreds of antimicrobials belonging to various classes, and the different RND efflux systems in one species are altogether able to export a wide set of substrates ranging from lipophilic to amphiphilic molecules and finally to toxic divalent cations [89–91]. Several examples of pumps belonging to this family are AcrAB-TolC and AcrAD-TolC of *E. coli* and MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM of *P. aeruginosa* [47, 92]. They span the entire periplasmic space from the cytoplasmic membrane to the OM by forming tripartite efflux complex systems [93] comprising an RND transporter protein (e.g., AcrB) embedded in the inner (cytoplasmic) membrane, a periplasmic adaptor protein (a.k.a. membrane fusion protein; e.g., AcrA) located in the periplasmic space, and an OM protein resembling a long helical tunnel (e.g., TolC). Recently, a small cytoplasmic membrane protein known as AcrZ was found to be associated with AcrB of *E. coli* and might have potential role in enhancing the transport activity of AcrB for specific antimicrobials like chloramphenicol, puromycin, and tetracycline [94]. No trace of any such protein or its homologs has been found in other RND transporters. Du et al. [95] presented a pseudo-atomic structure of this entire tripartite system AcrABZ-TolC to explain the quaternary organization and key domain interactions and also proposed a cooperative process for channel assembly and opening. The RND transporter protein structurally resembles a jellyfish with each protomer comprising a total of three domains

[96, 97]: (i) TMD consisting of 12 α-helices embedded in the inner cytoplasmic membrane is the region where energy conversion via proton conduction takes place; (ii) pore (porter) domain in the periplasm where substrate recruitment and transport mainly occur; and (iii) OM protein docking domain also in the periplasm, which couples the RND transporter to the OM protein or to the hexameric assembly of membrane fusion proteins in the constituted pump.

The drug-binding sites for the RND family are within the periplasmic domain of the protein, in contrast to other MDR pumps discussed above [8] as evident from the drug-bound structures in the asymmetrical trimer configuration [97–99]. It has been postulated that the "resting state" of these transporters (i.e., the structure in the absence of substrates) corresponds to a symmetric structure in which each monomer assumes the same conformation, while the presence of substrates or inhibitors triggers conformational changes leading to an asymmetric configuration [49] (also see Chap. 1 of this book). The latter is characterized by three possible structures of each monomer in the trimer, which were indeed interpreted as reaction cycle intermediates: *loose* (a.k.a. "access") in which substrates become associated by loosely binding to a proximal (access) pocket, *tight* (a.k.a. "binding") in which substrates bind tightly to a more distal (deep) binding pocket, and *open* (a.k.a. "extrusion") which corresponds to the drug-released state of a functionally rotating mechanism (see Fig. 30.1; also see Fig. 1.6 of Chap. 1) [90, 98, 104–106]. A recent study [107] has put forward the hypothesis that high molecular mass substrates (and low molecular mass dimers as well [108]) are actually recognized by the proximal pocket of the *loose* monomer, while low molecular mass compounds are recognized by the distal pocket of the *tight* monomer instead.

According to the functional rotation mechanism, a concerted but not necessarily synchronous [105, 109] cycling of the monomers occurs through any of the asymmetric states: *loose*, *tight*, *open*, and back to *loose*. During a complete functional cycle, occlusions and constrictions inside the pore domain propagate from external gates toward the central funnel, driving the unidirectional transport of substrate ("peristaltic pump mechanism" [104]). In other terms, the substrate would gain access to the pore domain of the transporter via the *loose* and/or *tight* monomer, either from open clefts in the periplasm or through grooves between helices at the interface between pore and TMD [110, 111]. The substrate would then get accommodated into a large binding pocket when the monomer assumes the *tight* state and moved out toward the TolC docking domain upon a subsequent change to the *open* conformation.

These tripartite multidrug transporters are highly efficient in creating detectable resistance to antimicrobials as they export the drug substrates directly from the periplasm or the inner leaflet of the cytoplasmic membrane into the external medium, making the reentry of drugs through the low permeable OM cumbersome. The efficiency of RND pumps is synergistically associated with the presence and ability of single-component pumps located in the cytoplasmic membrane to flush out substrates from the cytoplasm [73, 74].

The past decade has seen numerous structural studies performed on various representative proteins from all five aforementioned multidrug transporter families (see

Chaps. 1, 2, 3, and 4), thus providing valuable data forming the foundation to explore similarities and differences in drug recognition and drug export mechanisms and for the future therapeutic inhibition of these transporters [112].

## 30.5 Inhibition Strategies for Efflux Pumps

Since active drug efflux plays a major role in intrinsic and acquired drug resistance in Gram-negative bacteria, inactivation of such pumps may open up a wide arena of possibilities for better antimicrobial adjuvant therapy. This strategy has several advantages [113] such as (i) elevation of the intracellular concentration of antimicrobials, (ii) reduction in the efflux-mediated intrinsic bacterial resistance, (iii) reversal of the acquired resistance associated with efflux pump overexpression, (iv) reduction in the frequency of emergence of highly resistant mutant strains by reducing the adaption time for development of additional mechanisms of resistance like target-based mutations [114], and (v) prevention of the export of endogenous microbial virulence factors, thus inhibiting microbial invasiveness [115, 116].

To revive the activity of an efflux-susceptible drug, efflux-mediated MDR can be inactivated by any of the following methods:

(i) Targeting the regulatory network involving activators and repressors that control the expression of efflux pumps [117] (e.g., altering the expression of AcrB from *Salmonella enterica* [11]; regulating efflux pump expression in *P. aeruginosa* [118–122]; targeting local repressor EmrR to alter the expression of EmrAB, a MFS transporter in *E. coli* [123]).

(ii) Altering the molecular design of existing susceptible antimicrobials to make them devoid of the chemophore recognized by efflux pump (e.g., chemically modified taxol escapes the action of P-glycoprotein [124]; tigecycline circumvents MFS pumps specific for tetracyclines [125]; telithromycin bypasses MefA/E and AcrAB systems [126]; among fluoroquinolones, gatifloxacin, levofloxacin, and moxifloxacin are not affected by NorA and PmrA pumps [127]). However, resistance against new compounds developed by this strategy was described shortly after their deployment [8].

(iii) Blocking the cytoplasmic membrane proteins with a high affinity competitively binding substrate (an EPI) to trap the efflux pump in an inactive conformation. These EPIs are clinically significant as they help evade antimicrobial resistance by inhibiting these pumps, reverse the acquired resistance associated with the overexpression of efflux pumps, and also suppress the emergence of mutations leading to resistance [10, 128–131] (e.g., the EPI of AcrB and MexB pumps, phenylalanine-arginine β-naphthylamide [PAβN]) [132]. However, toxicity issues have withheld these EPIs from clinical applications [10], although new compounds are being developed that have minimal toxicity but strong inhibitory effects on AcrB [133–135].

(iv) Depleting proton gradient to deprive the cytoplasmic membrane proteins of the motive force needed to work (e.g., carbonyl cyanide *m*-chlorophenylhydrazone [136], valinomycin, dinitrophenol, and phenothiazines such as promethazine [113, 128, 131, 137]; verapamil inhibits the MDR pumps of cancer cells and parasites in addition to improving the activity of tobramycin; reserpine inhibits the activity of Gram-positive efflux pumps Bmr and NorA [138]). However, these inhibitors affect the entire energetics of bacterial and also of eukaryotic cells, which makes them less attractive for clinical implication [116, 139].

(v) Specifically in the case of RND efflux pumps, the following approaches may disrupt the pump functionality: preventing the functional tripartite assembly formation by targeting protein-protein interfaces (e.g., designed ankyrin repeat proteins [DARPins] inhibited AcrAB-TolC formation by obstructing AcrA and AcrB interaction [140]); disrupting the interaction between AcrB and AcrZ (e.g., the absence of AcrZ diminished the substrate pool of AcrAB-TolC [94]); and blocking the exit duct (the OMP) (e.g., indole derivatives designed based on the structure of TolC prevented the opening of the channel [141], large cations targeting the negatively charged aspartate-rich entrance of TolC in *E. coli* [142]).

Among these different strategies to combat efflux-mediated MDR, inhibition of efflux pumps is considered to be a viable one [113], because a single potent inhibitor capable of competitively binding to a pump and preventing expulsion of its substrate antimicrobials could in principle also bind and block other MDR pumps overlapping in their substrate profiles [133, 135, 143]. In addition to revitalizing the therapeutic potential of the antimicrobials, these EPIs could also contribute to antibacterial action by hindering the transport of compounds needed for the normal growth and/or maintenance of the microorganism.

Numerous studies to date have guided our understanding of the structural and functional aspects of drug transporters at a molecular level and have also unveiled several fundamental concepts regarding their substrate binding and transport (for recent reviews, see, e.g., [9, 47, 49, 88, 91, 109, 144, 145]). These findings are useful for the rational design of inhibitors that can competitively bind to the efflux pumps and prevent the efflux of their substrate antimicrobials (and are also useful for the design of more efficient drugs that can escape efflux pumps) [49, 112].

A compound must satisfy the following criteria as postulated by Lomovskaya et al. [10] to qualify as an ideal clinically significant EPI: (i) It must potentiate the activity of antimicrobials in resistant strains expressing functional drug efflux pump. (ii) It must not have a significant effect on susceptible strains lacking the specific drug efflux pump. Moreover, the inhibitor should be free of any pharmacological activity on eukaryotic cells [146]. (iii) It must not potentiate the activity of antimicrobials that are not effluxed. (iv) It must increase the level of accumulation and decrease the level of extrusion of substrates of the efflux pump. (v) It must not permeabilize the OM; and (vi) It must not affect the proton gradient across the cytoplasmic membrane.

## 30.6 Computational Studies on Drug Efflux Pumps and Their Inhibitors

### *30.6.1 Role of Molecular Modeling in Drug Discovery*

Our understanding of the structural aspects of MDR pumps from crystallographic structures has been significant but not sufficient to fruitfully assist structure-based drug design. To address mechanistic knowledge gaps, computational techniques [147] are a great resource as they can highlight functional dynamics of biological systems. In particular, molecular docking and MD are increasingly being used both for rationalizing existing data and for various predictions, for instance, about drug recognition and binding, translocation mechanisms, and structural relations with the surrounding environment using three-dimensional structures.

Molecular docking tries to mimic the natural course of interaction of the ligand and its receptor via a lowest energy pathway and in doing so offers a great benefit in quick and efficient prediction of binding modes of small molecules to proteins but not necessarily the accurate binding energies [148–150]. The absence of a protein X-ray crystal structure creates a major hindrance in studies of ligand-protein interactions, but development of a suitable homology model [151–153] of a target protein that can then be used for molecular docking and other structure-based studies could provide an alternative approach. Pharmacophoric studies based on the receptor or the ligand are important to identify the most common structural moieties that contribute to drug recognition and can be exploited in drug design [154–156].

MD simulation is a powerful technique that can provide atomic level descriptions of molecular systems with high temporal resolution. It is also often employed to validate the stability of homology models. Atomic level simulations in the scale of several hundred nanoseconds are routinely performed to obtain a detailed insight into conformational changes and free energies of interactions and at the same time to identify drug-binding locations, translocation processes, and interactions with the surrounding lipid bilayer [157]. From such techniques, a normal-mode analysis and a functional mode analysis of the protein movements allow the comparison between simulations of the apo and holo structures, with one or several molecules inside the drug-binding pocket [158]. This could lead to the identification of the movements intimately related with the translocation process, aiming for a better understanding of the first steps of the efflux mechanism [159]. Recently, the use of "coarse-grained" simulations, where four atoms are typically combined into one particle, and biased MD simulations has increased dramatically. Such simulations allow sampling of large conformational changes that would normally be inaccessible because of the large free energy barriers between such conformations and the consequent limitations due to the lack of computational time [160–163].

Computational methods with improved algorithms now provide a higher level of understanding of biochemistry for better design of compounds and in economical use of the available biological/chemical resources [158, 159, 164]. Various computational studies on the EPIs of multidrug transporters, mainly those specific to RND

and ABC transporters, have been reported. Most of these studies are focused on identification of effective EPIs by high-throughput screening of compound databases and determination of the mechanism of inhibitor function by analyzing the molecular level inhibitor-pump interactions and the coupled conformational changes occurring in the transporters. In the following, we describe examples from the relevant literature for several families of MDR efflux pumps.

### *30.6.2 RND Pumps*

Inhibitors of these pumps include both medicinal plant extracts [165, 166] and synthetic compounds [134, 167–169]. Regarding the former compounds, two such successful studies have been reported so far where potent EPIs have been identified by *in silico* screening of natural compound databases. In one case, Ohene-Agyei et al. [165] employed molecular docking based screening to predict the bioactivity of plant compounds as effective inhibitors of AcrB by comparing with the known EPI, PAβN. They identified six compounds from docking results, of which plumbagin and nordihydroguaiaretic acid were found to be promising EPIs based on further efflux inhibition assays. In another case, Aparna et al. [166] obtained hits which are non-substrates of AcrB and MexB efflux proteins by using high-throughput virtual screening of an in-house database of phytochemicals and subsequently performing an exclusion-based filtering with the common pharmacophore models generated on the basis of known substrates of these pumps. These hits were then subjected to extra-precision docking against AcrB and MexB proteins and *in vitro* efflux inhibitory activity testing which eventually helped in the identification of lanatoside C and daidzein as promising EPIs effective for use in combination therapy against drug-resistant strains of *P. aeruginosa* and *E. coli*.

Takatsuka et al. [167] performed molecular docking of about 30 compounds (including substrates and inhibitors) to predict their interaction with the binding pocket of the *tight* protomer of AcrB as a means of understanding the substrate selectivity of AcrB. This study showed the presence of two large sites within the binding pocket, of which a narrow groove at one end of the pocket was preferred to a wide cave present at the other end of the pocket (Fig. 30.2). This docking study was validated by competition assays using nitrocefin efflux and covalent labeling of Phe615Cys mutant AcrB with fluorescein-5-maleimide, which also confirmed that the presumed groove binders competed against each other but not with the cave binders.

The first EPI-based MD simulation on RND transporters was reported by Vargiu and Nikaido [168], who examined the binding of nine substrates, two inhibitors, and two non-substrates to the distal pocket of AcrB in the presence of explicit water. They found that both the inhibitors (PAβN and 1-(1-naphtylmethyl)-piperazine [NMP]) bind to the lower part of the distal pocket that is rich in phenylalanine residues. After identification of the binding site for the inhibitor D13-9001 by X-ray crystallography, this pocket was also named a “hydrophobic trap” [132]. Though PAβN and NMP showed a fairly high binding affinity to the distal pocket of AcrB in docking study [167], both inhibitors slightly moved out of the pocket toward the

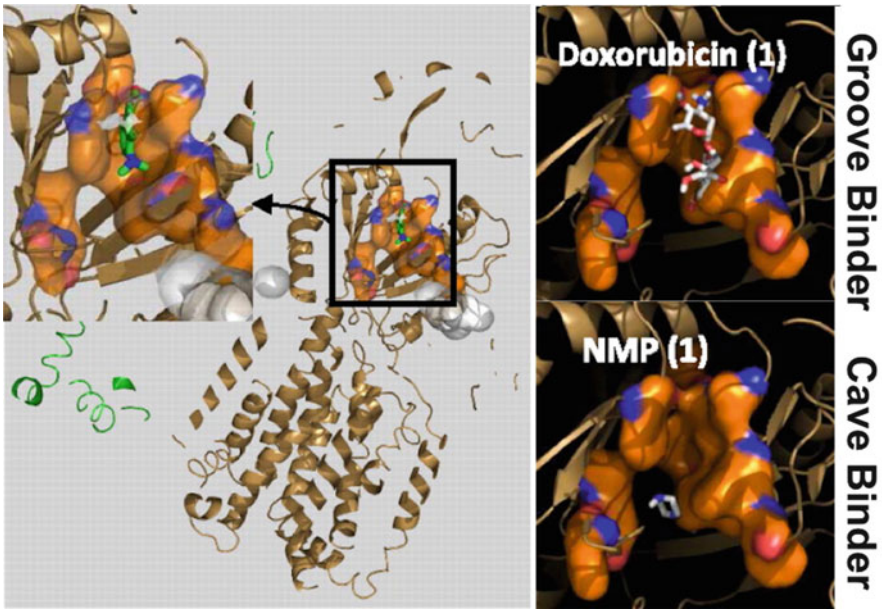



**Fig. 30.2** (*Left*) Side view of the binding protomer of AcrB asymmetric trimer with the proximal portion clipped away to reveal the binding pocket shown as surface with carbons in *orange*. The co-crystallized minocycline (PDB code 2DRD) is shown as *green* sticks. (*Inset*) Enlargement of the binding pocket (*right*), predicted binding site of inhibitor 1-(1-naphtylmethyl)-piperazine (NMP) (cave binder) and substrate doxorubicin (groove binder) (Modified from Takatsuka et al. [167])

G-loop and straddled it during the course of the MD simulation (Fig. 30.3). This provided a possible explanation for the mechanisms of inhibition by PAβN and NMP. These inhibitors when bound to AcrB likely reduce the flexibility of the G-loop which is important for the smooth translocation of substrates between the proximal pocket and the distal pocket. This proposed explanation agrees well with findings from the recent experimental [108, 132] and MD simulation studies [170] of the Gly616Pro and Gly619Pro AcrB mutants, where mutations in the G-loop impaired the drug export [99, 107, 108].

A recent work by Vargiu et al. [169] identified the underlying molecular mechanism of inhibition of MBX2319 (a pyranopyridine EPI potent against RND pumps of the *Enterobacteriaceae* species) by comparing it with that of other inhibitors like D13-9001, PAβN, and NMP by molecular docking and MD simulations. They observed that D13-9001 and MBX2319 bound more tightly than the typical substrate minocycline to the distal pocket of the *tight* monomer. The binding mode of MBX2319 was comparable to that of doxorubicin in the Phe610Ala variant of AcrB [171, 172]. By binding to the lower part of the distal pocket in the *tight* protomer of AcrB, this inhibitor interacts in a manner similar to that of the hydrophobic portion of D13-9001 [132] with hydrophobic phenylalanine-rich cage branching off from the substrate-translocation channel (Fig. 30.4) [169]. Investigation of the minocycline

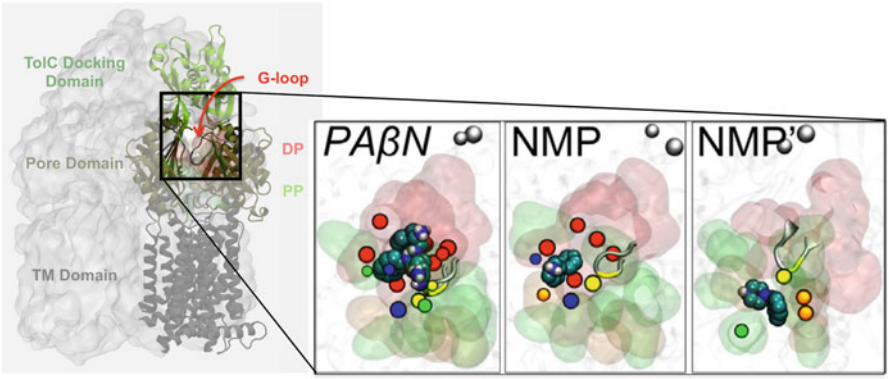



**Fig. 30.3** Comparison among different binding modes of PAβN and NMP to the distal and proximal (NMP') binding pockets of AcrB showing straddling of G-loop by these inhibitors. Ligands are shown with spheres colored according to atom types (with nonpolar hydrogens removed). The distal pocket (DP), proximal pocket (PP), and the PC1/PC2 subdomain Cleft are shown with transparent *red*, *green*, and *orange* surfaces, respectively, while the G-loop is shown in *gray* cartoon. Residues within 3.5 Å from the ligand are shown as colored beads (*red*, *green*, *orange*, and *yellow* for those of DP, PP, Cleft, and G-loop, respectively). The residues common to both the pockets are colored *blue*. Residues defining the exit gate (far away from the ligand) are shown as *gray* beads (Modified from Refs. [49, 168])

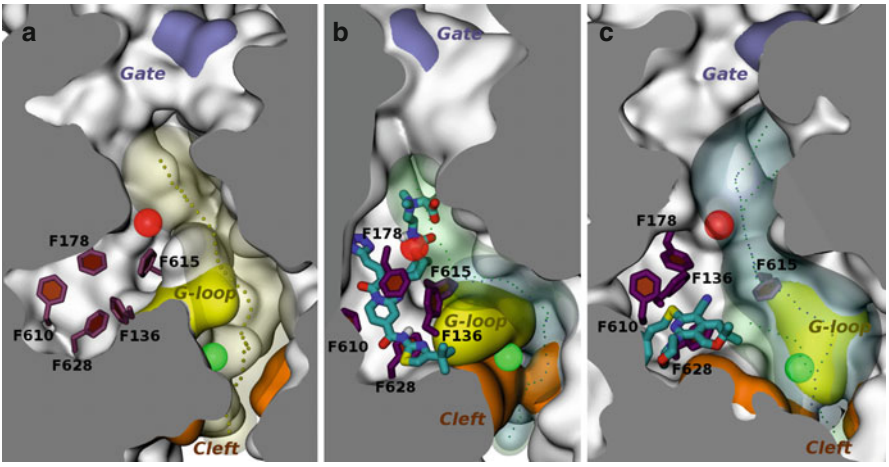
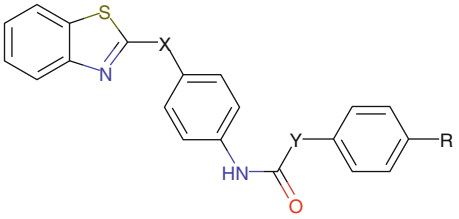



**Fig. 30.4** Position of inhibitors D13-9001 (**b**) and MBX2319 (**c**) with respect to the hydrophobic trap in *tight* protomer of AcrB, as found in representative average structures of the complexes from MD simulations. The channel found in AcrB free of ligands (**a**) is also shown for reference. Ligands are shown in *thick sticks*; protein is shown with the molecular surface colored in *orange*, *yellow*, and *ice blue* at the PC1/PC2 cleft, the G-loop tip, and the exit gate, respectively, and *white* elsewhere (Adapted from Vargiu et al. [169])

(substrate) binding to such AcrB-inhibitor complexes supports the hypothesis that all these inhibitors (except D13-9001) could function by competitive binding. As MBX2319 neither contains any charged groups nor can utilize common specific channels to penetrate across the OM of *P. aeruginosa*, it does not remarkably inhibit efflux in this species [47, 133].

Continuing efforts are in progress to develop more potent broad-spectrum EPIs to effectively counter the efflux-mediated MDR in bacteria. One such success story is the development of potent derivatives of MBX2319, some of which are 30 times more potent than the original inhibitor, based on the potentiation of levofloxacin and piperacillin [134]. A very recent study combining the data from cellular, X-ray crystallographic analyses, and MD simulations allowed to unveil the molecular basis for pyranopyridine-based inhibition of AcrB [173]. Particularly, in this study [173], a soluble version of AcrB was engineered (essentially identical to the truncated model of AcrB previously used in MD simulations [168]), highly congruent in structure with the periplasmic part of the full-length protein and capable of binding substrates and potent inhibitors. All of the pyranopyridines included in the work [173] bind within the hydrophobic trap forming extensive hydrophobic interactions. Moreover, the increasing potency of improved inhibitors correlates with the formation of a delicate protein- and water-mediated hydrogen bond network. In addition to giving insights into the mechanism for AcrB efflux inhibition, the setup employed in this new study [173] provides a molecular platform for the development of novel combinational therapies against pathogenic *Enterobacteriaceae*.

One another successful development of EPIs was recently reported by Yilmaz et al. [174], where a modified docking approach named core-constrained docking was employed to identify and characterize the binding site of two-substituted benzothiazoles as potential EPIs with the ability to restore the antibacterial activity of ciprofloxacin in an AcrAB-TolC overexpressing mutant. In the core-constrained docking method, the ligand scaffold is constrained during the initial minimization and conformer generation stages, but is given flexibility during the final refinement stages. Among the compounds experimentally tested by them, BSN-004, BSN-006, and BSN-023 (Fig. 30.5) topped the list with clinically significant EPI activity and were found to bind similar to the co-crystallized AcrB substrates ciprofloxacin, minocycline, and doxorubicin in the distal pocket of the *binding* monomer. Also, the




| *Compound code* | *R* | *X* | *Y* |
|---|---|---|---|
| BSN-004 | $OCH_3$ | - | $CH_2$ |
| BSN-006 | $CH_3$ | - | $CH_2$ |
| BSN-023 | H | - | $C_2H_4$ |

**BSN coded 2-substituted benzothiazoles**

**Fig. 30.5** Chemical structures of BSN coded 2-substituted benzothiazoles

higher calculated binding energies of BSN-006 and BSN-023 compared to that of ciprofloxacin indicated their possible role as competitive inhibitors in contrast to BSN-004, which with its lower binding energy might act as an uncompetitive inhibitor by simple steric hindrance.

Another very recent study was published by Nikaido and coworkers, who for the first time determined quantitatively the efflux transport kinetics of the EPI PAβN and its homologs Ala, Arg, and Phe β-naphthylamides [175]. In addition, they assessed the behavior of PAβN and its homologs as modulators of nitrocefin efflux through AcrB. These experiments demonstrated that PAβN is efficiently pumped out by AcrB with a sigmoidal kinetics and is able to change the nitrocefin kinetics into a sigmoidal one too. Furthermore, computational modeling showed that modulatory activity of PAβN and its homologs on the efflux of other substrates can be rationalized by inspecting their mode of binding to AcrB. Overall, the data support the hypothesis that PAβN inhibits the efflux of AcrB substrates by both binding to the hydrophobic trap and by interfering with the binding of other drug substrates to the upper part of the binding pocket.

A review on the reports that have brought an advancement in our understanding of the mechanism of functioning of several potent EPIs against RND pumps has been recently authored by Opperman and Nguyen [135].

### *30.6.3 ABC Transporters*

The mammalian P-glycoprotein of the ABC transporters, whose bacterial homologs include MsbA and LmrA [176, 177], has been an efflux transporter of prime interest of this superfamily yielding valuable insights on the drug recognition and mechanism of transport [178]. We, therefore, have included important findings from this eukaryotic pump as they can be translated to their bacterial counterparts.

Vandevuer et al. [179] published the first computational study of EPIs of ABC transporters where they performed molecular docking of several first- and second-generation inhibitors (dexniguldipine, quinidine, quinine, S9788 [a lipophilic P-glycoprotein modulator], tamoxifen, and verapamil) of P-glycoprotein and evaluated the inhibitor interactions and binding positions in P-glycoprotein. The finding of different positions both for a single ligand and for different ligands corroborates the experimental evidence indicating the existence of multiple drug-binding sites. In agreement with a recently proposed pharmacophore model of P-glycoprotein ligands [180], several types of interactions including H-bonds, π-π, and cation-π were identified between P-glycoprotein and the docked ligands.

In order to identify the major differences in the behavior of substrate (colchicine and vinblastine) or inhibitor (latilagascene E, QZ59-SSS [cyclic-*tris*-(*S*)-valineselenazole], tariquidar, and verapamil) molecules inside the drug-binding pocket, Ferreira et al. [158] analyzed the type and number of contacts alongside the major residues involved in the ligand-protein interactions by docking and MD simulations. They found that with the exception of QZ59-SSS, all modulators exhibit a

higher number of nonbonded interactions especially with aromatic residues. They also observed that modulators frequently establish a higher number of simultaneous interactions. Their study identified several residues and at least two regions (Fig. 30.6 [Inset 1]) where interactions occur exclusively with modulators. The first region was located at the beginning of TMD6, comprising residues Leu328, Thr329,

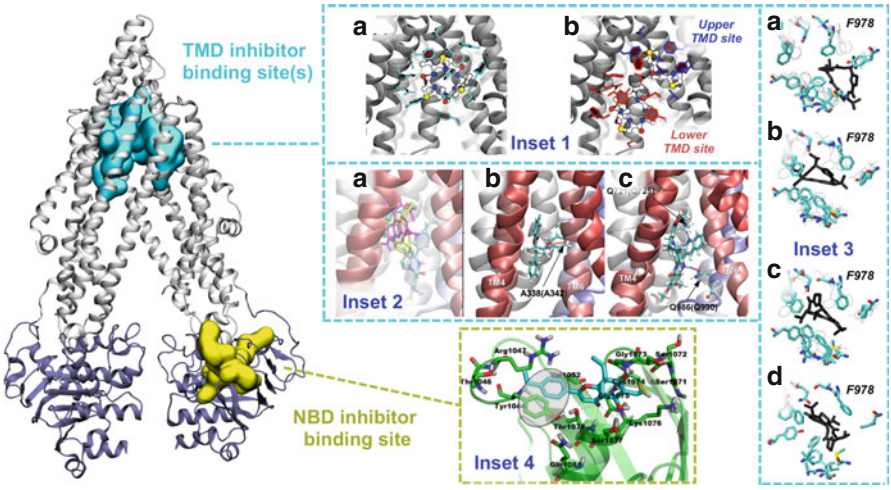



**Fig. 30.6** Binding sites found for various inhibitors within the TMD and NBD regions of ABC transporters. The structure of P-glycoprotein (PDB code 3G60) is shown on the *left* side with cartoon representation and the TMD and NDB domains colored *white* and *ice blue*, respectively. The overall positions of binding sites are shown by means of surface representation (*cyan* and *yellow* surfaces, respectively, for the TMD and NBD binding sites) of residues participating in the binding to several inhibitors. The *insets* on the *right* side show the magnified residue level details of the binding of a few inhibitors at that site reported from different studies: (*inset 1*) binding site interactions of QZ59-RRR (**a**) and QZ59-SSS molecules each in the lower (*red*) and upper (*blue*) sites (**b**) in the P-glycoprotein internal cavity as seen in the co-crystallized structures (PDB codes 3G60 and 3G61 [100]). The inhibitors are shown with CPK representation and colored according to the atom type (C, N, O, and S atoms are colored *white*, *blue*, *red*, and *yellow*, respectively), while the side chains of residues within 4 Å of the ligand are shown with *sticks*. (*Inset 2*) (**a**) The docked structure of the low energy conformation of inhibitor XR9576 (C, N, O, and H atoms are colored *cyan*, *blue*, *red*, and *white*, respectively) superimposed on substrate rhodamine 123 (*yellow sticks*) and another inhibitor GP240 (*pink sticks*). Inhibitors GP240 (**b**) and XR9576 (**c**) are stabilized by formation of H-bond with specific residues of P-glycoprotein (Obtained with permission from Elsevier [101]). (*Inset 3*) (**a–c**) Different binding modes of the inhibitor QZ59-RRR (*black sticks*) to the TMD drug-binding pocket of P-glycoprotein as obtained by docking the compound on three different conformations of the protein extracted from MD simulations [102]. The three conformations of the binding site are all shown in each subfigure, with thicker sticks (colored according to atom type as in Inset 2) referring to the conformation used for that specific docking run, and the thin lines used for the two remaining conformations. The crystal structure of mouse P-glycoprotein with QZ59-RRR bound is shown in (**d**) (Adapted from Wise [102]). (*Inset 4*) Docked pose of desmosdumotin (sticks colored according to atom type as in *Inset 2*) in NBD2 (*green* transparent helices) highlighting the stacking interactions realized between the phenyl group of the ligand and Tyr1044. Residues within 4 Å along with the observed hydrogen bonds (*red dashed lines*) are shown (Modified from Gadhe et al. [103])

Phe332, Ser333, and Leu335 and corresponds to an intersection of the QZ59-RRR (cyclic-*tris*-(*R*)-valineselenazole) and QZ59-SSS sites defined by Aller et al. [100]. The other region included residues from TMD7 (Ser725 and Phe728), TMD10 (Glu871, Met874, and Leu875), and TMD11 (Phe934). Several other residues (Met68, Phe332, Leu335, and Tyr946) were also identified to interact with at least three modulators but not with substrates. However, in the case of tariquidar, though a large number of interacting residues common to substrates vinblastine and colchicine were found suggesting a possible competition for these residues, additional interactions with Met874, Leu875, and Phe934 in TMD10/11 not observed for any of the substrates were also identified. This could be well correlated with the increased modulatory effect of tariquidar and may also guide the development of more selective and potent modulators.

In another study, Wise et al. [102] performed molecular docking and targeted MD simulations to evaluate the binding of inhibitors to P-glycoprotein. Ensemble docking was performed by taking 26 catalytically relevant non-redundant structures as receptors against 21 known transport ligands or inhibitors. In addition, the authors examined the transitions of the apo form from conformations that were wide open to the cytoplasm to transition state conformations that were wide open to the extracellular space and observed coupled movement of NBDs and TMDs that form the drug-binding cavities. NBDs showed pronounced twisting as the two domains approached each other, and this movement resulted in opening of the TMDs to the extracellular space as the ATP hydrolysis transition state was reached [102]. The largest movements of drug-binding site helices were observed for the pairs of helices 4/10 and helices 5/11. As the ATP hydrolysis transition state (fully opened outward conformation) approached, drug docking in the extracellular half of the transmembrane domains seemed to be destabilized as transport ligand exit gates opened to the extracellular space. The side chain of Phe978 (top of the binding site) was found to move out of the way in conformations close to the fully opened inward conformation thereby allowing QZ59-RRR analogue access to binding pocket (Figs. 30.5 and 30.6 [Inset 3]) [100, 102]. This supports the postulation of putative aromatic gating structures in the drug-binding sites of P-glycoprotein. The authors proposed that the destabilization of ligand binding in the extracellular half of the drug-binding site, coupled with denied access to ligand binding on the cytoplasmic side, would effectively force a release of the ligand to the extracellular space. They also suggested that there is no specific "inhibitor-binding site" located within the drug-binding domain of P-glycoprotein and that the mode of inhibition by these compounds, if binding occurs at the locations deduced from these docking studies, may be through the competition with substrate drugs.

In a similar study on EPIs for P-glycoprotein, Jara et al. [101] identified a common binding site for rhodamine 123 and modulators (derivatives of propafenone and XR9576 [tariquidar, one of the best modulators known at present]) with different modulation activity by performing molecular docking over the crystal structure of the mouse P-glycoprotein (Fig. 30.6 [Inset 2a]). The presence of a common binding site would suggest a competitive scheme for these inhibitors and the substrate rhodamine 123. Preliminary classical MD simulations on selected P-glycoprotein/

modulator complexes highlighted the importance of hydrophobic interactions and molecular flexibility of the modulator to fit the aromatic rings inside the TMD. It was found that the binding of two modulators (XR9576 and GP240 [a propafenone derivative]) was energetically more stable in P1 site than rhodamine 123 due to more favorable contributions of van der Waals interactions (hydrophobicity) and nonpolar solvation (Fig. 30.6 [Inset 2b, c]). Several interacting residues were found to be common to substrates and modulators in the region between transmembrane helices 4, 5, and 6 (Ser222, Ile306, Val338, Leu339, Ala342, and Phe343), with the aromatic residues contributing largely to the increase in the modulators' binding affinity. Binding of the inhibitor to this site could reduce the mobility of transmembrane helices (especially TM6) affecting the subsequent ATP hydrolysis. The interaction of TM12 (Val982) at a second site close to P1 was also observed with other inhibitors such as GP240. The molecular docking results in this study were concordant for some members of the GPxx family as reported by Klepsch et al. [181].

In order to investigate the role of P-glycoprotein flexibility in polyspecific drug binding, Liu et al. performed comparative MD simulations of inward-facing P-glycoprotein with/without inhibitor ligands (QZ59-RRR or QZ59-SSS) in explicit lipid and water environment [182]. They found that the flexibility of the binding pocket in P-glycoprotein, which is composed of the TMSs from both halves of P-glycoprotein, especially transmembrane helices 4, 5, and 6 and 10, 11, and 12, is essential for its polyspecific drug binding. Namely, while TM4 and TM5 are rigid and stabilize the whole structure, TM6 and TM12 show high flexibility, and the flexibility of the side chains of aromatic residues (Phe and Tyr) in the binding pocket allows them to form rotamers with different orientations, which is critical for the poly-specificity of the drug-binding cavity of P-glycoprotein. The authors found indeed the binding pocket of P-glycoprotein to be flexible and also to undergo ligand-induced conformational changes thus facilitating the residues lining the pocket to interact with multiple drugs. Finally, MD simulations illustrated the twisted conformational change of transmembrane regions in the outward-facing structure of P-glycoprotein, which might be important to export the substrate molecules, and the translational conformational change in the inward-facing structure, which regulates the opening/closing of the binding cavity of P-glycoprotein.

In order to have a comprehensive understanding of EPI action and conformational dynamics of desmosdumotin, an anticancer agent, Gadhe et al. explored its inhibition mechanism against P-glycoprotein (NBD2) by performing molecular docking and MD simulations [103]. Molecular docking showed that van der Waals and electrostatic interactions predominantly stabilize desmosdumotin binding to NBD2. MD simulations further indicated the involvement of Lys1076 and Ser1077 in hydrogen bonding and Tyr1044, Val1052, Gly1073, Cys1074, and Gly1075 in hydrophobic interactions. The π-π stacking hydrophobic interaction between the B-ring of desmosdumotin and side chain of Tyr1044 (encircled in gray in Fig. 30.6 [Inset 4]) identified in docking and stable during MD seems to be particularly important for inhibitor binding.

Recently, Ma et al. [183] carried out a systematic characterization and comparison of substrate (daunorubicin) and an inhibitor (QZ59-RRR and QZ59-SSS) effects on

NBD and TMD conformational dynamics using apo murine P-glycoprotein. Their simulation systems included the apo form of P-glycoprotein, the co-crystals with inhibitor QZ59 (QZ59-RRR and QZ59-SSS) bound (PDB codes 3G60 and 3G61, respectively [100]), and docking-generated complexes with the substrate daunorubicin bound to each of the two sites where inhibitor QZ59 was found. In six independent MD simulations of the apo protein embedded in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine bilayer, the authors observed an asymmetrical association of the NBDs where one of the two putative nucleotide-binding sites is further dissociated than the other, similar to what has been observed in other ABC transporter proteins. In the ligand bound complexes, this degree of association and the conformations of the nucleotide-binding site (Fig. 30.7) were dependent on the presence and the position of a substrate or an inhibitor bound in the TMD binding cavity. Namely, daunorubicin bound at the upper site triggers P-glycoprotein to undergo a closure event similar to that observed in apo simulations and also leads to the formation of the nucleotide-binding sites competent to bind ATP. The presence of an inhibitor (QZ59-RRR and QZ59-SSS) inside the drug-binding pocket kept the NBD site 2 open (maintaining crystallographic distances) with ATP-protein interaction energies significantly higher than the ones reported for substrates. This suggests that these inhibitors function by keeping the NBDs apart, thus preventing ATP hydrolysis. Moreover, the inhibitor QZ59-RRR exhibited higher affinities compared to that of the substrate, daunorubicin, owing to much more favorable van der Waals interactions.

Summarizing the findings of reference [183], a closure of the P-glycoprotein's internal binding pocket occurred only in the presence of the substrate bound at a

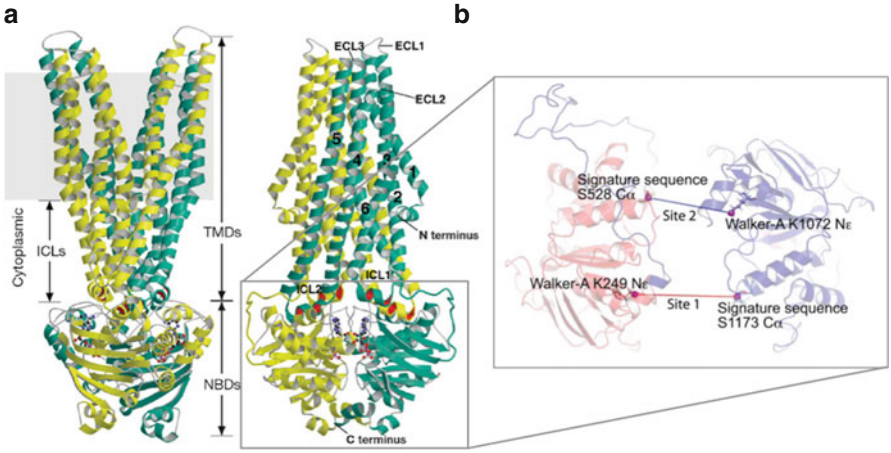



**Fig. 30.7** (**a**) The representative structure of ABC transporter Sav1866 with the subunits colored *yellow* and *turquoise* and highlighting the important domains. (**b**) Schematic illustration of the nucleotide-binding sites 1 and 2. Here, the N-terminal Walker A motif and the C-terminal signature sequence form "site 1," whereas the C-terminal Walker A motif and the N-terminal signature sequence form "site 2" (Adapted with permission from Macmillan Publisher Ltd: *Nature* [183, 184])

certain site in the binding pocket, while the inhibitor kept the two NBDs far apart. A greater number of ligand-protein interactions were formed by ligands docked at the lower site compared to the upper site during the unrestrained simulations, presumably reflecting the ability of P-glycoprotein to "wrap up" the ligands and suggesting a substrate-dependent behavior for P-glycoprotein efflux in which the ligand-induced fit seems to play a key role in drug recognition.

In another study aiming to elucidate the mechanism of translocation by and inhibition of P-glycoprotein, Prajapati et al. [185] modeled this transporter in three different catalytic states (inward open [IO] [NBDs are far apart], intermediate open [IIO], and outward open [OO] [NBDs are in close proximity]) and studied a total of 17 systems including eight substrates, eight inhibitors, and one without ligand by multi-targeted MD. Substantial details on the changes occurring in TMDs, the role of intracellular coupling helices, and the displacements and conformational changes in the residues lining drug-binding pocket during the catalytic transition of P-glycoprotein from its inward open to outward open state were traced. Though no distinct site for substrate and inhibitor binding was noticed, significant difference in substrate and inhibitor-binding interactions and stability was observed during the simulation from IO to OO state. The authors clearly showed how the loss of stable binding interactions destabilized the substrate binding in the active site of P-glycoprotein and dislodged it during the IO to OO transformation. In contrast, the inhibitors maintained stable interactions with drug-binding residues Phe303, Ile306, Phe343, Phe728, Ile868, Phe942, Thr945, and Ala985, posing possibility of inhibition of the conformational change in P-glycoprotein structure (Fig. 30.8).

In addition to studies on understanding the mechanism of action of existing inhibitors of ABC pumps, attempts have been made to improve their activity or to design new ones. One such study was performed by Tardia et al. who reported a new series of total 21 polymethoxy benzamides with the P-glycoprotein inhibitory activity. The submicromolar $IC_{50}$ level was reached through modulated lipophilicity of compounds and by establishment of an intramolecular hydrogen bond [186]. Eleven out of 21 of these compounds were active against both P-glycoprotein and MRP1. MD simulations and density functional theory calculations on these compounds advocated the presence of a unique conformation of the hit 4b (Fig. 30.9), which was characterized by a very stable intramolecular hydrogen bond. The authors claim that this conformational difference is the reason for the differential activities reported for the regioisomers 4a and 4b. They also state the strength of such intramolecular hydrogen bond interaction to be a sensitive parameter for soft modulation of the P-glycoprotein response as evident from 2,4,5-trimethoxybenzamide derivatives 3b, 4b, and 5b which display the highest activity and also the strongest intramolecular hydrogen bond.

Singh et al. [187] designed inhibitors of the transporter P-glycoprotein/MDR1 in *Leishmania*, responsible for the extrusion of miltefosine, a drug to treat leishmaniasis. Together with a series of activators of P4-ATPase protein to enhance import of miltefosine, a series of peptide inhibitors (Fig. 30.10a) of the P-glycoprotein-like ABC transporter were designed to overcome miltefosine resistance. The inhibitors were designed considering specificity to the target protein and also surface

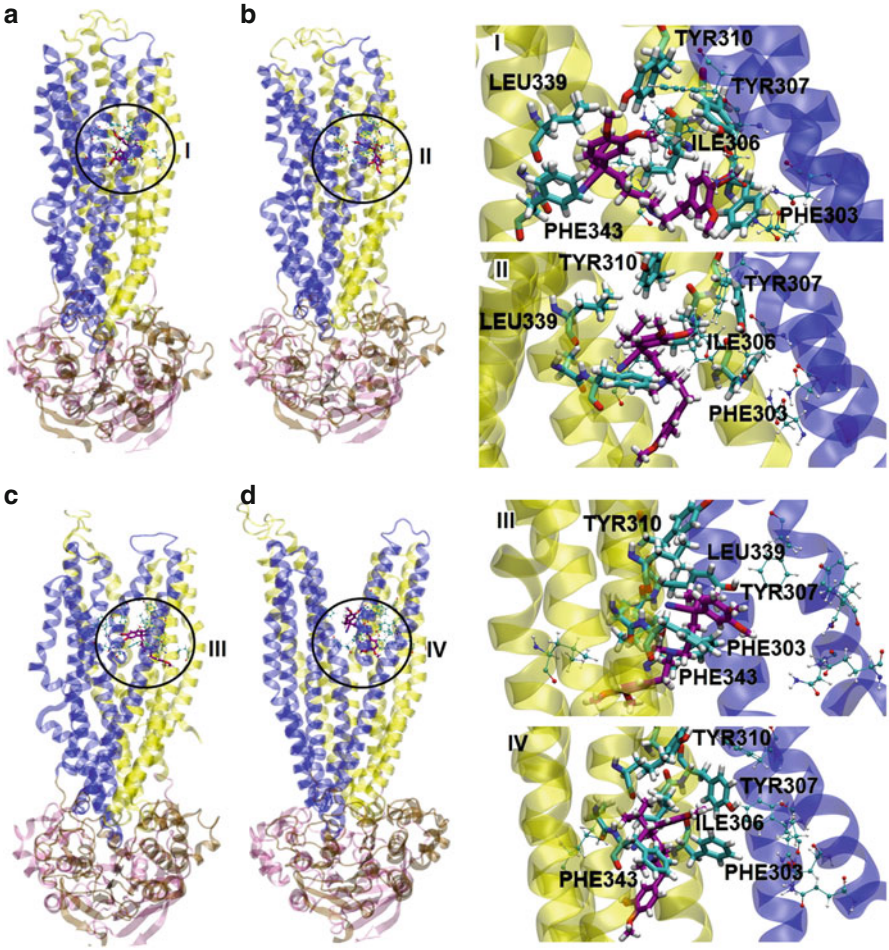



**Fig. 30.8** Changes in molecular interactions of verapamil (inhibitor) observed during multi-targeted molecular dynamics simulation; (**a**–**d**) represent the P-glycoprotein transition states: initial inward open, at starting of intermediate open, after intermediate open and outward open, respectively. The magnified images of corresponding encircled regions are shown as I, II, III, and IV, respectively (Obtained with permission from Elsevier [185])

orientation and flexibility. The molecular docking of these designed inhibitors confirmed the high affinity of inhibitor-9 having the sequence "QFIYYSAYALCFWY" and interacting with Asp1029, Ala1022, and His55 of the transporter (Fig. 30.10b). This study provided insights into the possibility of targeting P4-ATPase (important for the import of alkylphospholipid drugs into the parasite) and ABC transporters for improving the therapeutic efficiency of antileishmanial agents.

The results from the various computational studies on inhibitors of ABC pumps summarized above reflect the importance of copious nonbonded interactions to be formed by an inhibitor molecule to compete and establish itself strongly in the binding site of the pump, thenceforth impeding the required conformational changes for

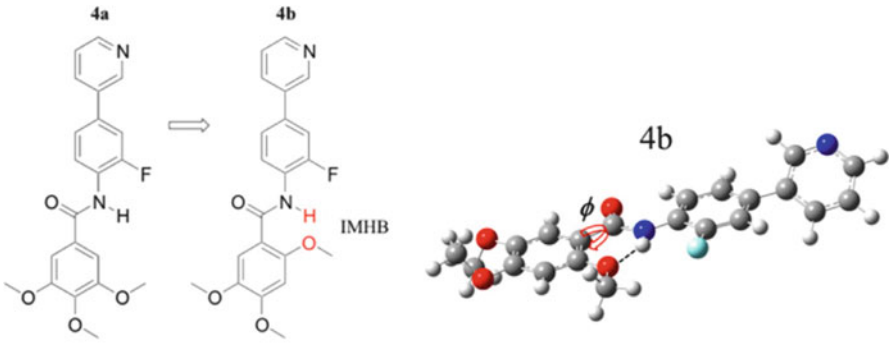



**Fig. 30.9** 2D structural representation of the regioisomers 4a and 4b highlighting the location of the intramolecular hydrogen bond (IMHB) (Obtained with permission from the American Chemical Society [186])

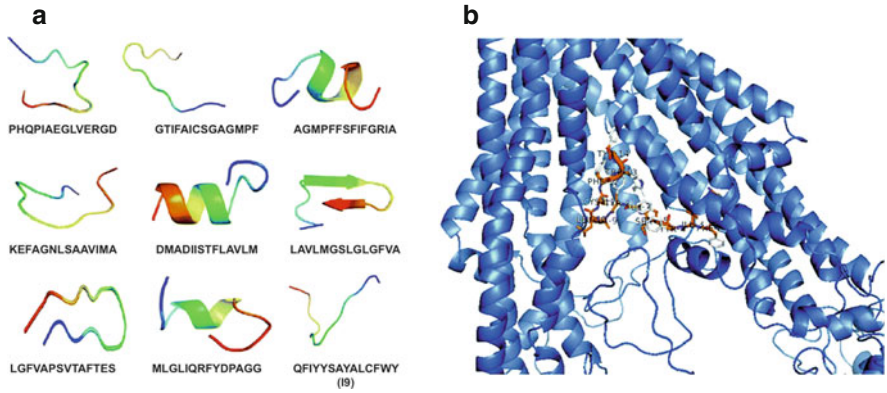



**Fig. 30.10** (**a**) Designed peptide inhibitors of the ABC transporters along with their amino acid sequence. (**b**) Docked complex of ABC transporter with peptide inhibitor I9 (Modified from Singh and Mandlik [187] with permission from the Royal Society of Chemistry)

substrate transport. These studies showcase the substantial success achieved so far in identification of putative binding sites of inhibitors, the interacting protein residues and the nature of predominant interactions, and the inhibition mechanism, all of which can be collectively exploited to develop novel and potent inhibitors as done by Tardia et al. [186] and Singh et al. [187].

### *30.6.4 MATE Transporters*

MD simulations were also employed in the study of EPIs for the MATE transporter NorA. The three isomeric hybrid compounds, SS14, SS14-M, and SS14-P, contain berberine, an antibacterial alkaloid known to be a substrate of NorA, fused at different positions of INF55 (5-nitro-2-phenylindole), an inhibitor of NorA. Tomkiewicz et al.

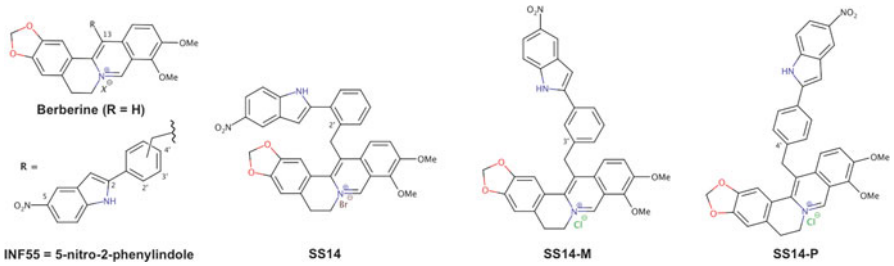



**Fig. 30.11** Chemical structures of berberine and INF55 moieties as well as the isomeric hybrid compounds (SS14, SS14-M, and SS14-P)

[188] analyzed the effects of varying the relative orientation of the antibacterial and EPI components in these three isomeric hybrids. They found that a subtle repositioning of the pump-blocking INF55 moiety in berberine-INF55 hybrids has a minimal effect on their antibacterial activities of the hybrids but has a significant effect on their inhibitory action against MDR pumps. Based on the experimental results, authors reported all three hybrids to have a very similar activity against *S. aureus* and *Caenorhabditis elegans*, though SS14 showed a slightly higher potency than its isomers against the wild-type and NorA-knockout strains. Also, the SS14 hybrid showed only a minor inhibitory effect on MDR pumps when compared to that of SS14-M and SS14-P. Through MD simulations, authors identified that the hybrid SS14 prefers to adopt a more compact globular conformation with the INF55 moiety folded back over the berberine unit, whereas in SS14-M and SS14-P, the INF55 moiety extends away from berberine (Fig. 30.11) [188]. The unique conformation for SS14 identified here may explain why it shows different bacterial cell uptake kinetics and reduced inhibitory effects on MDR pumps relative to those of SS14-M and SS14-P.

## 30.7 Concluding Remarks

MDR is an unavoidable natural phenomenon and needs to be effectively countered with highest priority to prevent the advent of a post-antibiotic era with untreatable life-threatening infections. Efflux transporters like those of the MFS members in Gram-positive bacteria and RND members in Gram-negative bacteria are the primary saviors in clinically important pathogens. These transporters, if inhibited, can hinder the normal physiology as well as the MDR exhibited by pathogens toward numerous drugs, eventually reviving the era of antibiotic treatable infections. The recent reports on computational studies significantly contributing toward the development of several EPIs of such transporter systems and a better understanding of the structure and function of efflux transporter have provided a positive ray of hope toward development of better EPIs and novel antimicrobial agents that can bypass efflux. It would definitely be interesting to improve these molecules to widen their spectrum of activity, even if attainment of a universal prokaryotic EPI might not be

pragmatic. In addition to focusing solely on the competitive inhibitors of the MDR pumps, scientists are now considering inhibition of transcription of the genes coding for efflux pumps or inhibition of other members of tripartite complexes as possible alternatives.

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## References

1. Fauci AS (2001) Infectious diseases: considerations for the 21st century. Clin Infect Dis 32:675–685. doi:10.1086/319235
2. World Health Organization (2014) Antimicrobial resistance: global report on surveillance. World Health Organization, Geneva
3. Howell L (2013) Global risks 2013: an initiative of the risk response network, 8th edn. World Economic Forum, Geneva
4. Butler MS, Cooper MA (2011) Antibiotics in the clinical pipeline in 2011. J Antibiot (Tokyo) 64:413–425. doi:10.1038/ja.2011.44
5. Nikaido H (1994) Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science 264:382–388. doi:10.1126/science.8153625
6. Poole K (2005) Efflux-mediated antimicrobial resistance. J Antimicrob Chemother 56: 20–51. doi:10.1093/jac/dki171
7. Piddock LJ (2006) Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev 19:382–402. doi:10.1128/CMR.19.2.382-402.2006
8. Nikaido H, Pagès JM (2012) Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. FEMS Microbiol Rev 36:340–363. doi:10.1111/j.1574-6976.2011.00290.x
9. Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ (2015) Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol 13:42–51. doi:10.1038/nrmicro3380
10. Lomovskaya O, Bostian KA (2006) Practical applications and feasibility of efflux pump inhibitors in the clinic – a vision for applied use. Biochem Pharmacol 71:910–918. doi:10.1016/j.bcp.2005.12.008
11. Blair JM, Richmond GE, Piddock LJ (2014) Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. Future Microbiol 9:1165–1177. doi:10.2217/fmb.14.66
12. Li X-Z, Nikaido H (2009) Efflux-mediated drug resistance in bacteria: an update. Drugs 69:1555–1623. doi:10.2165/11317030-000000000-00000
13. Zhang H, Wang Y-J, Zhang Y-K, Wang D-S, Kathawala RJ, Patel A, Talele TT, Chen Z-S et al (2014) AST1306, a potent EGFR inhibitor, antagonizes ATP-binding cassette subfamily G member 2-mediated multidrug resistance. Cancer Lett 350:61–68. doi:10.1016/j.canlet.2014.04.008
14. Martinez L, Arnaud O, Henin E, Tao H, Chaptal V, Doshi R, Andrieu T, Dussurgey S et al (2014) Understanding polyspecificity within the substrate-binding cavity of the human multidrug resistance P-glycoprotein. FEBS J 281:673–682. doi:10.1111/febs.12613
15. Kim J-Y, Henrichs S, Bailly A, Vincenzetti V, Sovero V, Mancuso S, Pollmann S, Kim D et al (2010) Identification of an ABCB/P-glycoprotein-specific inhibitor of auxin transport by chemical genomics. J Biol Chem 285:23309–23317. doi:10.1074/jbc.M110.105981

16. Michaelis M, Rothweiler F, Nerreter T, Van Rikxoort M, Sharifi M, Wiese M, Ghafourian T, Cinatl J (2014) Differential effects of the oncogenic BRAF inhibitor PLX4032 (vemurafenib) and its progenitor PLX4720 on ABCB1 function. J Pharm Pharm Sci 17:154–168
17. Singh DV, Godbole MM, Misra K (2013) A plausible explanation for enhanced bioavailability of P-gp substrates in presence of piperine: simulation for next generation of P-gp inhibitors. J Mol Model 19:227–238. doi:10.1007/s00894-012-1535-8
18. Liu D-L, Li Y-J, Yao N, Xu J, Chen Z-S, Yiu A, Zhang C-X, Ye W-C et al (2014) Acerinol, a cyclolanstane triterpenoid from *Cimicifuga acerina*, reverses ABCB1-mediated multidrug resistance in HepG2/ADM and MCF-7/ADR cells. Eur J Pharmacol 733:34–44. doi:10.1016/j.ejphar.2014.03.043
19. Klepsch F, Vasanthanathan P, Ecker GF (2014) Ligand and structure-based classification models for prediction of P-glycoprotein inhibitors. J Chem Inf Model 54:218–229. doi:10.1021/ci400289j
20. Zha W, Wang G, Xu W, Liu X, Wang Y, Zha BS, Shi J, Zhao Q et al (2013) Inhibition of P-glycoprotein by HIV protease inhibitors increases intracellular accumulation of berberine in murine and human macrophages. PLoS One 8:e54349. doi:10.1371/journal.pone.0054349
21. Zhao X-Q, Xie J-D, X-g C, Sim HM, Zhang X, Liang Y-J, Singh S, Talele TT et al (2012) Neratinib reverses ATP-binding cassette B1-mediated chemotherapeutic drug resistance *in vitro, in vivo*, and *ex vivo*. Mol Pharmacol 82:47–58. doi:10.1124/mol.111.076299
22. Hamm R, Sugimoto Y, Steinmetz H, Efferth T (2014) Resistance mechanisms of cancer cells to the novel vacuolar $H^+$-ATPase inhibitor archazolid B. Invest New Drugs 32:893–903. doi:10.1007/s10637-014-0134-1
23. Matsson P, Pedersen JM, Norinder U, Bergström CA, Artursson P (2009) Identification of novel specific and general inhibitors of the three major human ATP-binding cassette transporters P-gp, BCRP and MRP2 among registered drugs. Pharm Res 26:1816–1831. doi:10.1007/s11095-009-9896-0
24. Abdelfatah SA, Efferth T (2015) Cytotoxicity of the indole alkaloid reserpine from *Rauwolfia serpentina* against drug-resistant tumor cells. Phytomedicine 22:308–318. doi:10.1016/j.phymed.2015.01.002
25. Munagala S, Sirasani G, Kokkonda P, Phadke M, Krynetskaia N, Lu P, Sharom FJ, Chaudhury S et al (2014) Synthesis and evaluation of *Strychnos* alkaloids as MDR reversal agents for cancer cell eradication. Bioorg Med Chem 22:1148–1155. doi:10.1016/j.bmc.2013.12.022
26. Brewer FK, Follit CA, Vogel PD, Wise JG (2014) *In silico* screening for inhibitors of P-glycoprotein that target the nucleotide binding domains. Mol Pharmacol 86:716–726. doi:10.1124/mol.114.095414
27. Kim N, Shin J-M, No KT (2014) *In silico* study on the interaction between P-glycoprotein and its inhibitors at the drug binding pocket. Bull Korean Chem Soc 35:2317–2325. doi:10.5012/bkcs.2014.35.8.2317
28. Upadhyay HC, Dwivedi GR, Roy S, Sharma A, Darokar MP, Srivastava SK (2014) Phytol derivatives as drug resistance reversal agents. ChemMedChem 9:1860–1868. doi:10.1002/cmdc.201402027
29. Zeino M, Saeed ME, Kadioglu O, Efferth T (2014) The ability of molecular docking to unravel the controversy and challenges related to P-glycoprotein – a well-known, yet poorly understood drug transporter. Invest New Drugs 32:618–625. doi:10.1007/s10637-014-0098-1
30. Silva R, Carmo H, Vilas-Boas V, Barbosa DJ, Palmeira A, Sousa E, Carvalho F, de Lourdes Bastos M et al (2014) Colchicine effect on P-glycoprotein expression and activity: *in silico* and *in vitro* studies. Chem Biol Interact 218:50–62. doi:10.1016/j.cbi.2014.04.009
31. Kathawala RJ, Chen J-J, Zhang Y-K, Wang Y-J, Patel A, Wang D-S, Talele TT, Ashby CR et al (2014) Masitinib antagonizes ATP-binding cassette subfamily G member 2-mediated multidrug resistance. Int J Oncol 44:1634–1642. doi:10.3892/ijo.2014.2341
32. Dwivedi GR, Upadhyay HC, Yadav DK, Singh V, Srivastava SK, Khan F, Darmwal NS, Darokar MP (2014) 4-Hydroxy-α-tetralone and its derivative as drug resistance reversal agents in multi drug resistant *Escherichia coli*. Chem Biol Drug Des 83:482–492. doi:10.1111/cbdd.12263

33. Tajima Y, Nakagawa H, Tamura A, Kadioglu O, Satake K, Mitani Y, Murase H, Regasini LO et al (2014) Nitensidine A, a guanidine alkaloid from *Pterogyne nitens*, is a novel substrate for human ABC transporter ABCB1. Phytomedicine 21:323–332. doi:10.1016/j.phymed.2013.08.024
34. Tan W, Mei H, Chao L, Liu T, Pan X, Shu M, Yang L (2013) Combined QSAR and molecule docking studies on predicting P-glycoprotein inhibitors. J Comput Aided Mol Des 27:1067–1073. doi:10.1007/s10822-013-9697-8
35. Ferreira RJ, Ferreira M-JU, dos Santos DJ (2013) Molecular docking characterizes substrate-binding sites and efflux modulation mechanisms within P-glycoprotein. J Chem Inf Model 53:1747–1760. doi:10.1021/ci400195v
36. Tiwari AK, Sodani K, C-l D, Abuznait AH, Singh S, Xiao Z-J, Patel A, Talele TT et al (2013) Nilotinib potentiates anticancer drug sensitivity in murine ABCB1-, ABCG2-, and ABCC10-multidrug resistance xenograft models. Cancer Lett 328:307–317. doi:10.1016/j.canlet.2012.10.001
37. Chufan EE, Kapoor K, Sim H-M, Singh S, Talele TT, Durell SR, Ambudkar SV (2013) Multiple transport-active binding sites are available for a single substrate on human P-glycoprotein (ABCB1). PLoS One 8:e82463. doi:10.1371/journal.pone.0082463
38. Kanaoka S, Kimura Y, Fujikawa M, Nakagawa Y, Ueda K, Akamatsu M (2013) Substrate recognition by P-glycoprotein efflux transporters: structure-ATPase activity relationship of diverse chemicals and agrochemicals. J Pest Sci 38:112–122. doi:10.1584/jpestics.D13-022
39. Zhang D-M, Shu C, Chen J-J, Sodani K, Wang J, Bhatnagar J, Lan P, Ruan Z-X et al (2012) BBA, a derivative of 23-hydroxybetulinic acid, potently reverses ABCB1-mediated drug resistance *in vitro* and *in vivo*. Mol Pharm 9:3147–3159. doi:10.1021/mp300249s
40. Dolghih E, Bryant C, Renslo AR, Jacobson MP (2011) Predicting binding to P-glycoprotein by flexible receptor docking. PLoS Comput Biol 7:e1002083. doi:10.1371/journal.pcbi.1002083
41. Kalia NP, Mahajan P, Mehra R, Nargotra A, Sharma JP, Koul S, Khan IA (2012) Capsaicin, a novel inhibitor of the NorA efflux pump, reduces the intracellular invasion of *Staphylococcus aureus*. J Antimicrob Chemother 67:2401–2408. doi:10.1093/jac/dks232
42. Xiao Z-P, Wang X-D, Wang P-F, Zhou Y, Zhang J-W, Zhang L, Zhou J, Zhou S-S et al (2014) Design, synthesis, and evaluation of novel fluoroquinolone–flavonoid hybrids as potent antibiotics against drug-resistant microorganisms. Eur J Med Chem 80:92–100. doi:10.1016/j.ejmech.2014.04.037
43. George AM (1996) Multidrug resistance in enteric and other Gram-negative bacteria. FEMS Microbiol Lett 139:1–10. doi:10.1111/j.1574-6968.1996.tb08172.x
44. Pagès J-M, Amaral L (2009) Mechanisms of drug efflux and strategies to combat them: challenging the efflux pump of Gram-negative bacteria. Biochim Biophys Acta 1794:826–833. doi:10.1016/j.bbapap.2008.12.011
45. Upadhyay R (2011) Emergence of drug resistance in microbes, its dissemination and target modification of antibiotics: a life threatening problem to human society. Int J Pharm Biol Res 2:119–126
46. Utsui Y, Yokota T (1985) Role of an altered penicillin-binding protein in methicillin-and cephem-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 28:397–403. doi:10.1128/AAC.28.3.397
47. Li X-Z, Plésiat P, Nikaido H (2015) The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. Clin Microbiol Rev 28:337–418. doi:10.1128/CMR.00117-14
48. Sapunaric FM, Aldema-Ramos M, McMurry LM (2005) Tetracycline resistance: efflux, mutation, and other mechanisms. In: White DG, Alekshun MN, McDermot PF (eds) Frontiers in antimicrobial resistance, a tribute to Stuart B. Levy. ASM Press, Washington, DC, pp 3–18
49. Ruggerone P, Murakami S, Pos KM, Vargiu AV (2013) RND efflux pumps: structural information translated into function and inhibition mechanisms. Curr Top Med Chem 13:3079–3100. doi:10.2174/15680266113136660220
50. Blair JM, Bavro VN, Ricci V, Modi N, Cacciotto P, Kleinekathfer U, Ruggerone P, Vargiu AV et al (2015) AcrB drug-binding pocket substitution confers clinically relevant resistance

and altered substrate specificity. Proc Natl Acad Sci U S A 112:3511–3516. doi:10.1073/pnas.1419939112
51. Piddock LJ (2006) Multidrug-resistance efflux pumps – not just for resistance. Nat Rev Microbiol 4:629–636. doi:10.1038/nrmicro1464
52. Rosner JL, Martin RG (2009) An excretory function for the *Escherichia coli* outer membrane pore TolC: upregulation of *marA* and *soxS* transcription and Rob activity due to metabolites accumulated in *tolC* mutants. J Bacteriol 191:5283–5292. doi:10.1128/JB.00507-09
53. Paul S, Alegre KO, Holdsworth SR, Rice M, Brown JA, McVeigh P, Kelly SM, Law CJ (2014) A single-component multidrug transporter of the major facilitator superfamily is part of a network that protects *Escherichia coli* from bile salt stress. Mol Microbiol 92:872–884. doi:10.1111/mmi.12597
54. Guelfo JR, Rodriguez-Rojas A, Matic I, Blazquez J (2010) A MATE-family efflux pump rescues the *Escherichia coli* 8-oxoguanine-repair-deficient mutator phenotype and protects against $H_2O_2$ killing. PLoS Genet 6:e1000931. doi:10.1371/journal.pgen.1000931
55. Bogomolnaya LM, Andrews KD, Talamantes M, Maple A, Ragoza Y, Vazquez-Torres A, Andrews-Polymenis H (2013) The ABC-type efflux pump MacAB protects *Salmonella enterica* serovar Typhimurium from oxidative stress. mBio 4:e00630-13. doi:10.1128/mBio.00630-13
56. Poole K (2012) Stress responses as determinants of antimicrobial resistance in Gram-negative bacteria. Trends Microbiol 20:227–234. doi:10.1016/j.tim.2012.02.004
57. Podnecky NL, Rhodes KA, Schweizer HP (2015) Efflux pump-mediated drug resistance in *Burkholderia*. Front Microbiol 6:305. doi:10.3389/fmicb.2015.00305
58. Baugh S, Phillips CR, Ekanayaka AS, Piddock LJ, Webber MA (2014) Inhibition of multidrug efflux as a strategy to prevent biofilm formation. J Antimicrob Chemother 69:673–681. doi:10.1093/jac/dkt420
59. Matsumura K, Furukawa S, Ogihara H, Morinaga Y (2011) Roles of multidrug efflux pumps on the biofilm formation of *Escherichia col*i K-12. Biocontrol Sci 16:69–72. doi:10.4265/bio.16.69
60. Saier MH Jr, Reddy VS, Tamang DG, Vastermark A (2014) The transporter classification database. Nucleic Acids Res 42:D251–D258. doi:10.1093/nar/gkt1097
61. Ren Q, Chen K, Paulsen IT (2007) TransportDB: a comprehensive database resource for cytoplasmic membrane transport systems and outer membrane channels. Nucleic Acids Res 35:D274–D279. doi:10.1093/nar/gkl925
62. Eswaran J, Koronakis E, Higgins MK, Hughes C, Koronakis V (2004) Three's company: component structures bring a closer view of tripartite drug efflux pumps. Curr Opin Struct Biol 14:741–747. doi:10.1016/j.sbi.2004.10.003
63. Du D, van Veen HW, Murakami S, Pos KM, Luisi BF (2015) Structure, mechanism and cooperation of bacterial multidrug transporters. Curr Opin Struct Biol 33:76–91. doi:10.1016/j.sbi.2015.07.015
64. Higgins CF (2001) ABC transporters: physiology, structure and mechanism – an overview. Res Microbiol 152:205–210. doi:10.1016/S0923-2508(01)01193-7
65. Shapiro AB, Fox K, Lam P, Ling V (1999) Stimulation of P-glycoprotein-mediated drug transport by prazosin and progesterone. Eur J Biochem 259:841–850. doi:10.1046/j.1432-1327.1999.00098.x
66. Martin C, Berridge G, Higgins CF, Mistry P, Charlton P, Callaghan R (2000) Communication between multiple drug binding sites on P-glycoprotein. Mol Pharmacol 58:624–632. doi:10.1124/mol.58.3.624
67. Higgins CF, Linton KJ (2004) The ATP switch model for ABC transporters. Nat Struct Mol Biol 11:918–926. doi:10.1038/nsmb836
68. Lu M, Symersky J, Radchenko M, Koide A, Guo Y, Nie R, Koide S (2013) Structures of a $Na^+$-coupled, substrate-bound MATE multidrug transporter. Proc Natl Acad Sci U S A 110:2099–2104. doi:10.1073/pnas.1219901110
69. Schuldiner S (2012) Undecided membrane proteins insert in random topologies. Up, down and sideways: it does not really matter. Trends Biochem Sci 37:215–219. doi:10.1016/j.tibs.2012.02.006

70. Martin C, Berridge G, Mistry P, Higgins C, Charlton P, Callaghan R (2000) Drug binding sites on P-glycoprotein are altered by ATP binding prior to nucleotide hydrolysis. Biochemistry 39:11901–11906. doi:10.1021/bi000559b
71. McDevitt CA, Crowley E, Hobbs G, Starr KJ, Kerr ID, Callaghan R (2008) Is ATP binding responsible for initiating drug translocation by the multidrug transporter ABCG2? FEBS J 275:4354–4362. doi:10.1111/j.1742-4658.2008.06578.x
72. Yan N (2013) Structural advances for the major facilitator superfamily (MFS) transporters. Trends Biochem Sci 38:151–159. doi:10.1016/j.tibs.2013.01.003
73. Lee A, Mao W, Warren MS, Mistry A, Hoshino K, Okumura R, Ishida H, Lomovskaya O (2000) Interplay between efflux pumps may provide either additive or multiplicative effects on drug resistance. J Bacteriol 182:3142–3150. doi:10.1128/JB.182.11.3142-3150.2000
74. Tal N, Schuldiner S (2009) A coordinated network of transporters with overlapping specificities provides a robust survival strategy. Proc Natl Acad Sci U S A 106:9051–9056. doi:10.1073/pnas.0902400106
75. Lewinson O, Adler J, Sigal N, Bibi E (2006) Promiscuity in multidrug recognition and transport: the bacterial MFS Mdr transporters. Mol Microbiol 61:277–284. doi:10.1111/j.1365-2958.2006.05254.x
76. Fluman N, Ryan CM, Whitelegge JP, Bibi E (2012) Dissection of mechanistic principles of a secondary multidrug efflux protein. Mol Cell 47:777–787. doi:10.1016/j.molcel.2012.06.018
77. Nikaido H, Zgurskaya HI (1999) Antibiotic efflux mechanisms. Curr Opin Infect Dis 12:529–536
78. Kuroda T, Tsuchiya T (2009) Multidrug efflux transporters in the MATE family. Biochim Biophys Acta 1794:763–768. doi:10.1016/j.bbapap.2008.11.012
79. He X, Szewczyk P, Karyakin A, Evin M, Hong WX, Zhang Q, Chang G (2010) Structure of a cation-bound multidrug and toxic compound extrusion transporter. Nature 467:991–994. doi:10.1038/nature09408
80. Tanaka Y, Hipolito CJ, Maturana AD, Ito K, Kuroda T, Higuchi T, Katoh T, Kato HE et al (2013) Structural basis for the drug extrusion mechanism by a MATE multidrug transporter. Nature 496:247–251. doi:10.1038/nature12014
81. Paulsen IT, Skurray RA, Tam R, Saier MH Jr, Turner RJ, Weiner JH, Goldberg EB, Grinius LL (1996) The SMR family: a novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs. Mol Microbiol 19:1167–1175. doi:10.1111/j.1365-2958.1996.tb02462.x
82. Schuldiner S (2009) EmrE, a model for studying evolution and mechanism of ion-coupled transporters. Biochim Biophys Acta 1794:748–762. doi:10.1016/j.bbapap.2008.12.018
83. Pornillos O, Chen Y-J, Chen AP, Chang G (2005) X-ray structure of the EmrE multidrug transporter in complex with a substrate. Science 310:1950–1953. doi:10.1126/science.1119776
84. Chen YJ, Pornillos O, Lieu S, Ma C, Chen AP, Chang G (2007) X-ray structure of EmrE supports dual topology model. Proc Natl Acad Sci U S A 104:18999–19004. doi:10.1073/pnas.0709387104
85. Korkhov VM, Tate CG (2008) Electron crystallography reveals plasticity within the drug binding site of the small multidrug transporter EmrE. J Mol Biol 377:1094–1103. doi:10.1016/j.jmb.2008.01.056
86. Morrison EA, DeKoster GT, Dutta S, Vafabakhsh R, Clarkson MW, Bahl A, Kern D, Ha T et al (2011) Antiparallel EmrE exports drugs by exchanging between asymmetric structures. Nature 481:45–50. doi:10.1038/nature10703
87. Rotem D, Schuldiner S (2004) EmrE, a multidrug transporter from *Escherichia coli*, transports monovalent and divalent substrates with the same stoichiometry. J Biol Chem 279:48787–48793. doi:10.1074/jbc.M408187200
88. Venter H, Mowla R, Ohene-Agyei T, Ma S (2015) RND-type drug efflux pumps from Gram-negative bacteria: molecular mechanism and inhibition. Front Microbiol 6:377. doi:10.3389/fmicb.2015.00377
89. Saier M, Tam R, Reizer A, Reizer J (1994) Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. Mol Microbiol 11:841–847. doi:10.1111/j.1365-2958.1994.tb00362.x

90. Nikaido H (1996) Multidrug efflux pumps of Gram-negative bacteria. J Bacteriol 178:5853–5859
91. Dreier J, Ruggerone P (2015) Interaction of antibacterial compounds with RND efflux pumps in Pseudomonas aeruginosa. Front Microbiol 6:660. doi:10.3389/fmicb.2015.00660
92. Sulavik MC, Houseweart C, Cramer C, Jiwani N, Murgolo N, Greene J, DiDomenico B, Shaw KJ et al (2001) Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. Antimicrob Agents Chemother 45:1126–1136. doi:10.1128/AAC.45.4.1126-1136.2001
93. Symmons MF, Bokma E, Koronakis E, Hughes C, Koronakis V (2009) The assembled structure of a complete tripartite bacterial multidrug efflux pump. Proc Natl Acad Sci U S A 106:7173–7178. doi:10.1073/pnas.0900693106
94. Hobbs EC, Yin X, Paul BJ, Astarita JL, Storz G (2012) Conserved small protein associates with the multidrug efflux pump AcrB and differentially affects antibiotic resistance. Proc Natl Acad Sci U S A 109:16696–16701. doi:10.1073/pnas.1210093109
95. Du D, Wang Z, James NR, Voss JE, Klimont E, Ohene-Agyei T, Venter H, Chiu W et al (2014) Structure of the AcrAB-TolC multidrug efflux pump. Nature 509:512–515. doi:10.1038/nature13205
96. Murakami S, Nakashima R, Yamashita E, Yamaguchi A (2002) Crystal structure of bacterial multidrug efflux transporter AcrB. Nature 419:587–593. doi:10.1038/nature01050
97. Sennhauser G, Bukowska MA, Briand C, Grutter MG (2009) Crystal structure of the multidrug exporter MexB from *Pseudomonas aeruginosa*. J Mol Biol 389:134–145. doi:10.1016/j.jmb.2009.04.001
98. Murakami S, Nakashima R, Yamashita E, Matsumoto T, Yamaguchi A (2006) Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. Nature 443:173–179. doi:10.1038/nature05076
99. Nakashima R, Sakurai K, Yamasaki S, Nishino K, Yamaguchi A (2011) Structures of the multidrug exporter AcrB reveal a proximal multisite drug-binding pocket. Nature 480:565–569. doi:10.1038/nature10641
100. Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT et al (2009) Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 323:1718–1722. doi:10.1126/science.1168750
101. Jara GE, Vera DMA, Pierini AB (2013) Binding of modulators to mouse and human multidrug resistance P-glycoprotein. A computational study. J Mol Graph Model 46:10–21. doi:10.1016/j.jmgm.2013.09.001
102. Wise JG (2012) Catalytic transitions in the human MDR1 P-glycoprotein drug binding sites. Biochemistry 51:5125–5141. doi:10.1021/bi300299z
103. Gadhe CC, Kothandan G, Joo Cho S (2013) *In silico* study of desmosdumotin as an anticancer agent: homology modeling, docking and molecular dynamics simulation approach. Anti-Cancer Agents Med Chem 13:1636–1644. doi:10.2174/18715206113139990302
104. Seeger MA, Schiefner A, Eicher T, Verrey F, Diederichs K, Pos KM (2006) Structural asymmetry of AcrB trimer suggests a peristaltic pump mechanism. Science 313:1295–1298. doi:10.1126/science.1131542
105. Seeger MA, Diederichs K, Eicher T, Brandstatter L, Schiefner A, Verrey F, Pos KM (2008) The AcrB efflux pump: conformational cycling and peristalsis lead to multidrug resistance. Curr Drug Targets 9:729–749. doi:10.2174/138945008785747789
106. Murakami S (2008) Multidrug efflux transporter, AcrB – the pumping mechanism. Curr Opin Struct Biol 18:459–465. doi:10.1016/j.sbi.2008.06.007
107. Nakashima R, Sakurai K, Yamasaki S, Nishino K, Yamaguchi A (2011) Structures of the multidrug exporter AcrB reveal a proximal multisite drug-binding pocket. Nature 480:565–569. doi:10.1038/nature10641
108. Eicher T, Cha HJ, Seeger MA, Brandstatter L, El-Delik J, Bohnert JA, Kern WV, Verrey F et al (2012) Transport of drugs by the multidrug transporter AcrB involves an access

and a deep binding pocket that are separated by a switch-loop. Proc Natl Acad Sci U S A 109:5687–5692. doi:10.1073/pnas.1114944109
109. Pos KM (2009) Drug transport mechanism of the AcrB efflux pump. Biochim Biophys Acta 1794:782–793. doi:10.1016/j.bbapap.2008.12.015
110. Nikaido H, Basina M, Nguyen V, Rosenberg EY (1998) Multidrug efflux pump AcrAB of *Salmonella typhimurium* excretes only those β-lactam antibiotics containing lipophilic side chains. J Bacteriol 180:4686–4692
111. Husain F, Bikhchandani M, Nikaido H (2011) Vestibules are part of the substrate path in the multidrug efflux transporter AcrB of *Escherichia coli*. J Bacteriol 193:5847–5849. doi:10.1128/JB.05759-11
112. Wong K, Ma J, Rothnie A, Biggin PC, Kerr ID (2014) Towards understanding promiscuity in multidrug efflux pumps. Trends Biochem Sci 39:8–16. doi:10.1016/j.tibs.2013.11.002
113. Zechini B, Versace I (2009) Inhibitors of multidrug resistant efflux systems in bacteria. Recent Pat Antiinfect Drug Discov 4:37–50. doi:10.2174/157489109787236256
114. Sun J, Deng Z, Yan A (2014) Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. Biochem Biophys Res Commun 453:254–267. doi:10.1016/j.bbrc.2014.05.090
115. Hirakata Y, Kondo A, Hoshino K, Yano H, Arai K, Hirotani A, Kunishima H, Yamamoto N et al (2009) Efflux pump inhibitors reduce the invasiveness of *Pseudomonas aeruginosa*. Int J Antimicrob Agents 34:343–346. doi:10.1016/j.ijantimicag.2009.06.007
116. Bhardwaj AK, Mohanty P (2012) Bacterial efflux pumps involved in multidrug resistance and their inhibitors: rejuvinating the antimicrobial chemotherapy. Recent Pat Antiinfect Drug Discov 7:73–89. doi:10.2174/157489112799829710
117. Grkovic S, Brown MH, Skurray RA (2002) Regulation of bacterial drug export systems. Microbiol Mol Biol Rev 66:671–701. doi:10.1128/MMBR.66.4.671-701.2002
118. Wilke MS, Heller M, Creagh AL, Haynes CA, McIntosh LP, Poole K, Strynadka NC (2008) The crystal structure of MexR from *Pseudomonas aeruginosa* in complex with its antirepressor ArmR. Proc Natl Acad Sci U S A 105:14832–14837. doi:10.1073/pnas.0805489105
119. Starr LM, Fruci M, Poole K (2012) Pentachlorophenol induction of the *Pseudomonas aeruginosa mexAB-oprM* efflux operon: involvement of repressors NalC and MexR and the antirepressor ArmR. PLoS One 7:e32684. doi:10.1371/journal.pone.0032684
120. Hay T, Fraud S, Lau CH, Gilmour C, Poole K (2013) Antibiotic inducibility of the *mexXY* multidrug efflux operon of *Pseudomonas aeruginosa*: involvement of the MexZ anti-repressor ArmZ. PLoS One 8:e56858. doi:10.1371/journal.pone.0056858
121. Purssell A, Poole K (2013) Functional characterization of the NfxB repressor of the *mexCD-oprJ* multidrug efflux operon of *Pseudomonas aeruginosa*. Microbiology 159:2058–2073. doi:10.1099/mic.0.069286-0
122. Lau CH, Hughes D, Poole K (2014) MexY-promoted aminoglycoside resistance in *Pseudomonas aeruginosa*: involvement of a putative proximal binding pocket in aminoglycoside recognition. mBio 5:e01068–14. doi:10.1128/mBio.01068-14
123. Lomovskaya O, Lewis K, Matin A (1995) EmrR is a negative regulator of the *Escherichia coli* multidrug resistance pump EmrAB. J Bacteriol 177:2328–2334
124. Rice A, Liu Y, Michaelis ML, Himes RH, Georg GI, Audus KL (2005) Chemical modification of paclitaxel (Taxol) reduces P-glycoprotein interactions and increases permeation across the blood-brain barrier *in vitro* and *in situ*. J Med Chem 48:832–838. doi:10.1021/jm040114b
125. Chopra I (2002) New developments in tetracycline antibiotics: glycylcyclines and tetracycline efflux pump inhibitors. Drug Resist Updat 5:119–125. doi:10.1016/S1368-7646(02)00051-1
126. Chollet R, Chevalier J, Bryskier A, Pagès JM (2004) The AcrAB-TolC pump is involved in macrolide resistance but not in telithromycin efflux in *Enterobacter aerogenes* and *Escherichia coli*. Antimicrob Agents Chemother 48:3621–3624. doi:10.1128/AAC.48.9.3621-3624.2004
127. Hooper DC (2000) Mechanisms of action and resistance of older and newer fluoroquinolones. Clin Infect Dis 31(Suppl 2):S24–S28. doi:10.1086/314056

128. Pagès JM, Masi M, Barbe J (2005) Inhibitors of efflux pumps in Gram-negative bacteria. Trends Mol Med 11:382–389. doi:10.1016/j.molmed.2005.06.006
129. Marquez B (2005) Bacterial efflux systems and efflux pumps inhibitors. Biochimie 87:1137–1147. doi:10.1016/j.biochi.2005.04.012
130. Lynch AS (2006) Efflux systems in bacterial pathogens: an opportunity for therapeutic intervention? An industry view. Biochem Pharmacol 71:949–956. doi:10.1016/j.bcp.2005.10.021
131. Mahamoud A, Chevalier J, Alibert-Franco S, Kern WV, Pagès J-M (2007) Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. J Antimicrob Chemother 59:1223–1229. doi:10.1093/jac/dkl493
132. Nakashima R, Sakurai K, Yamasaki S, Hayashi K, Nagata C, Hoshino K, Onodera Y, Nishino K et al (2013) Structural basis for the inhibition of bacterial multidrug exporters. Nature 500:102–106. doi:10.1038/nature12300
133. Opperman TJ, Kwasny SM, Kim HS, Nguyen ST, Houseweart C, D'Souza S, Walker GC, Peet NP et al (2014) Characterization of a novel pyranopyridine inhibitor of the AcrAB efflux pump of *Escherichia coli*. Antimicrob Agents Chemother 58:722–733. doi:10.1128/AAC.01866-13
134. Nguyen ST, Kwasny SM, Ding X, Cardinale SC, McCarthy CT, Kim H-S, Nikaido H, Peet NP et al (2015) Structure–activity relationships of a novel pyranopyridine series of Gram-negative bacterial efflux pump inhibitors. Bioorg Med Chem 23:2024–2034. doi:10.1016/j.bmc.2015.03.016
135. Opperman TJ, Nguyen ST (2015) Recent advances toward a molecular mechanism of efflux pump inhibition. Front Microbiol 6:421. doi:10.3389/fmicb.2015.00421
136. Viveiros M, Jesus A, Brito M, Leandro C, Martins M, Ordway D, Molnar AM, Molnar J et al (2005) Inducement and reversal of tetracycline resistance in *Escherichia coli* K-12 and expression of proton gradient-dependent multidrug efflux pump genes. Antimicrob Agents Chemother 49:3578–3582. doi:10.1128/AAC.49.8.3578-3582.2005
137. Martins M, Dastidar SG, Fanning S, Kristiansen JE, Molnar J, Pagès JM, Schelz Z, Spengler G et al (2008) Potential role of non-antibiotics (helper compounds) in the treatment of multidrug-resistant Gram-negative infections: mechanisms for their direct and indirect activities. Int J Antimicrob Agents 31:198–208. doi:10.1016/j.ijantimicag.2007.10.025
138. Li X-Z, Nikaido H (2004) Efflux-mediated drug resistance in bacteria. Drugs 64:159–204. doi:10.2165/00003495-200464020-00004
139. Kourtesi C, Ball AR, Huang Y-Y, Jachak SM, Vera DMA, Khondkar P, Gibbons S, Hamblin MR et al (2013) Microbial efflux systems and inhibitors: approaches to drug discovery and the challenge of clinical implementation. Open Microbiol J 7:34–52. doi:10.2174/1874285801307010034
140. Tikhonova EB, Yamada Y, Zgurskaya HI (2011) Sequential mechanism of assembly of multidrug efflux pump AcrAB-TolC. Chem Biol 18:454–463. doi:10.1016/j.chembiol.2011.02.011
141. Zeng B, Wang H, Zou L, Zhang A, Yang X, Guan Z (2010) Evaluation and target validation of indole derivatives as inhibitors of the AcrAB-TolC efflux pump. Biosci Biotechnol Biochem 74:2237–2241. doi:10.1271/bbb.100433
142. Andersen C, Koronakis E, Hughes C, Koronakis V (2002) An aspartate ring at the TolC tunnel entrance determines ion selectivity and presents a target for blocking by large cations. Mol Microbiol 44:1131–1139. doi:10.1046/j.1365-2958.2002.02898.x
143. Chevalier J, Mulfinger C, Garnotel E, Nicolas P, Davin-Regli A, Pagès JM (2008) Identification and evolution of drug efflux pump in clinical *Enterobacter aerogenes* strains isolated in 1995 and 2003. PLoS One 3:e3203. doi:10.1371/journal.pone.0003203
144. Yamaguchi A, Nakashima R, Sakurai K (2015) Structural basis of RND-type multidrug exporters. Front Microbiol 6:327. doi:10.3389/fmicb.2015.00327
145. Du D, van Veen HW, Luisi BF (2015) Assembly and operation of bacterial tripartite multidrug efflux pumps. Trends Microbiol 23:311–319. doi:10.1016/j.tim.2015.01.010

146. Van Bambeke F, Lee VJ (2006) Inhibitors of bacterial efflux pumps as adjuvants in antibiotic treatments and diagnostic tools for detection of resistance by efflux. Recent Pat Antiinfect Drug Discov 1:157–175. doi:10.2174/157489106777452692
147. Schwede T, Peitsch M (2008) Computational structural biology: methods and applications. World Scientific Publishing Co. Pte. Ltd., Singapore
148. Halperin I, Ma B, Wolfson H, Nussinov R (2002) Principles of docking: an overview of search algorithms and a guide to scoring functions. Proteins 47:409–443. doi:10.1002/prot.10115
149. van Dijk AD, Boelens R, Bonvin AM (2005) Data-driven docking for the study of biomolecular complexes. FEBS J 272:293–312. doi:10.1111/j.1742-4658.2004.04473.x
150. van Dijk AD, Bonvin AM (2006) Solvated docking: introducing water into the modelling of biomolecular complexes. Bioinformatics 22:2340–2347. doi:10.1093/bioinformatics/btl395
151. Martí-Renom MA, Stuart AC, Fiser A, Sánchez R, Melo F, Šali A (2000) Comparative protein structure modeling of genes and genomes. Ann Rev Biophys Biomol Struct 29:291–325. doi:10.1146/annurev.biophys.29.1.291
152. Šali A, Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol 234:779–815. doi:10.1006/jmbi.1993.1626
153. Eswar N, Webb B, Marti-Renom MA, Madhusudhan M, Eramian D, Shen M, Pieper U, Sali A (2006) Comparative protein structure modeling using Modeller. Curr Protoc Bioinformatics 15:5.6.1–5.6.30. doi:10.1002/0471250953.bi0506s15
154. Salam NK, Nuti R, Sherman W (2009) Novel method for generating structure-based pharmacophores using energetic analysis. J Chem Inf Model 49:2356–2368. doi:10.1021/ci900212v
155. Joseph-McCarthy D (1999) Computational approaches to structure-based ligand design. Pharmacol Ther 84:179–191. doi:10.1016/S0163-7258(99)00031-5
156. Lyne PD (2002) Structure-based virtual screening: an overview. Drug Discov Today 7: 1047–1055. doi:10.1016/S1359-6446(02)02483-2
157. Galeazzi R (2009) Molecular dynamics as a tool in rational drug design: current status and some major applications. Curr Comput Aided Drug Des 5:225–240. doi:10.2174/157340909789577847
158. Ferreira RJ, Ferreira M-JU, dos Santos DJ (2012) Insights on P-glycoprotein's efflux mechanism obtained by molecular dynamics simulations. J Chem Theory Comput 8:1853–1864. doi:10.1021/ct300083m
159. Ruggerone P, Vargiu AV, Collu F, Fischer N, Kandt C (2013) Molecular dynamics computer simulations of multidrug RND efflux pumps. Comput Struct Biotechnol J 5:e201302008. doi:10.5936/csbj.201302008
160. Schlitter J, Engels M, Krüger P (1994) Targeted molecular dynamics: a new approach for searching pathways of conformational transitions. J Mol Graph 12:84–89. doi:10.1016/0263-7855(94)80072-3
161. Izvekov S, Voth GA (2005) A multiscale coarse-graining method for biomolecular systems. J Phys Chem B 109:2469–2473. doi:10.1021/jp044629q
162. Takada S (2012) Coarse-grained molecular simulations of large biomolecules. Curr Opin Struct Biol 22:130–137. doi:10.1016/j.sbi.2012.01.010
163. Parkin J, Chavent M, Khalid S (2015) Molecular simulations of Gram-negative bacterial membranes: a vignette of some recent successes. Biophys J 109:461–468. doi:10.1016/j.bpj.2015.06.050
164. Collu F, Cascella M (2013) Multidrug resistance and efflux pumps: insights from molecular dynamics simulations. Curr Top Med Chem 13:3165–3183. doi:10.2174/15680266113136660224
165. Ohene-Agyei T, Mowla R, Rahman T, Venter H (2014) Phytochemicals increase the antibacterial activity of antibiotics by acting on a drug efflux pump. Microbiol Open 3:885–896. doi:10.1002/mbo3.212

166. Aparna V, Dineshkumar K, Mohanalakshmi N, Velmurugan D, Hopper W (2014) Identification of natural compound inhibitors for multidrug efflux pumps of *Escherichia coli* and *Pseudomonas aeruginosa* using *in silico* high-throughput virtual screening and *in vitro* validation. PLoS One 9:e101840. doi:10.1371/journal.pone.0101840
167. Takatsuka Y, Chen C, Nikaido H (2010) Mechanism of recognition of compounds of diverse structures by the multidrug efflux pump AcrB of *Escherichia coli*. Proc Natl Acad Sci U S A 107:6559–6565. doi:10.1073/pnas.1001460107
168. Vargiu AV, Nikaido H (2012) Multidrug binding properties of the AcrB efflux pump characterized by molecular dynamics simulations. Proc Natl Acad Sci U S A 109:20637–20642. doi:10.1073/pnas.1218348109
169. Vargiu AV, Ruggerone P, Opperman TJ, Nguyen ST, Nikaido H (2014) Molecular mechanism of MBX2319 inhibition of *Escherichia coli* AcrB multidrug efflux pump and comparison with other inhibitors. Antimicrob Agents Chemother 58:6224–6234. doi:10.1128/AAC.03283-14
170. Feng Z, Hou T, Li Y (2012) Unidirectional peristaltic movement in multisite drug binding pockets of AcrB from molecular dynamics simulations. Mol Biosyst 8:2699–2709. doi:10.1039/c2mb25184a
171. Vargiu AV, Collu F, Schulz R, Pos KM, Zacharias M, Kleinekathofer U, Ruggerone P (2011) Effect of the F610A mutation on substrate extrusion in the AcrB transporter: explanation and rationale by molecular dynamics simulations. J Am Chem Soc 133:10704–10707. doi:10.1021/ja202666x
172. Bohnert JA, Schuster S, Seeger MA, Fahnrich E, Pos KM, Kern WV (2008) Site-directed mutagenesis reveals putative substrate binding residues in the *Escherichia coli* RND efflux pump AcrB. J Bacteriol 190:8225–8229. doi:10.1128/JB.00912-08
173. Sjuts H, Vargiu AV, Kwasny SM, Nguyen ST, Kim H-S, Ding X, Ornik AR, Ruggerone P et al (2016) Molecular basis for inhibition of AcrB multidrug efflux pump by novel and powerful pyranopyridine derivatives. Proc Natl Acad Sci U S A 113:3509–3514. doi:10.1073/pnas.1602472113
174. Yilmaz S, Altinkanat-Gelmez G, Bolelli K, Guneser-Merdan D, Ufuk Over-Hasdemir M, Aki-Yalcin E, Yalcin I (2015) Binding site feature description of 2-substituted benzothiazoles as potential AcrAB-TolC efflux pump inhibitors in *E. coli*. SAR QSAR Environ Res 26:853–871. doi:10.1080/1062936X.2015.1106581
175. Kinana AD, Vargiu AV, May T, Nikaido H (2016) Aminoacyl β-naphthylamides as substrates and modulators of AcrB multidrug efflux pump. Proc Natl Acad Sci U S A 113:1405–1410. doi:10.1073/pnas.1525143113
176. van Veen HW, Venema K, Bolhuis H, Oussenko I, Kok J, Poolman B, Driessen AJ, Konings WN (1996) Multidrug resistance mediated by a bacterial homolog of the human multidrug transporter MDR1. Proc Natl Acad Sci U S A 93:10668–10672
177. Reuter G, Janvilisri T, Venter H, Shahi S, Balakrishnan L, van Veen HW (2003) The ATP binding cassette multidrug transporter LmrA and lipid transporter MsbA have overlapping substrate specificities. J Biol Chem 278:35193–35198. doi:10.1074/jbc.M306226200
178. Davidson AL, Dassa E, Orelle C, Chen J (2008) Structure, function, and evolution of bacterial ATP-binding cassette systems. Microbiol Mol Biol Rev 72:317–364. doi:10.1128/MMBR.00031-07
179. Vandevuer S, Van Bambeke F, Tulkens PM, Prévost M (2006) Predicting the three-dimensional structure of human P-glycoprotein in absence of ATP by computational techniques embodying crosslinking data: insight into the mechanism of ligand migration and binding sites. Proteins 63:466–478. doi:10.1002/prot.20892
180. Pajeva IK, Wiese M (2002) Pharmacophore model of drugs involved in P-glycoprotein multidrug resistance: explanation of structural variety (hypothesis). J Med Chem 45:5671–5686. doi:10.1021/jm020941h
181. Klepsch F, Chiba P, Ecker GF (2011) Exhaustive sampling of docking poses reveals binding hypotheses for propafenone type inhibitors of P-glycoprotein. PLoS Comput Biol 7:e1002036. doi:10.1371/journal.pcbi.1002036

182. Liu M, Hou T, Feng Z, Li Y (2013) The flexibility of P-glycoprotein for its poly-specific drug binding from molecular dynamics simulations. J Biomol Struct Dyn 31:612–629. doi:10.1080/07391102.2012.706079
183. Ma J, Biggin PC (2013) Substrate versus inhibitor dynamics of P-glycoprotein. Proteins 81:1653–1668. doi:10.1002/prot.24324
184. Dawson RJP, Locher KP (2006) Structure of a bacterial multidrug ABC transporter. Nature 443:180–185. doi:10.1038/nature05155
185. Prajapati R, Sangamwar AT (2014) Translocation mechanism of P-glycoprotein and conformational changes occurring at drug-binding site: insights from multi-targeted molecular dynamics. Biochim Biophys Acta 1838:2882–2898. doi:10.1016/j.bbamem.2014.07.018
186. Tardia P, Stefanachi A, Niso M, Stolfa DA, Mangiatordi GF, Alberga D, Nicolotti O, Lattanzi G et al (2014) Trimethoxybenzanilide-based P-glycoprotein modulators: an interesting case of lipophilicity tuning by intramolecular hydrogen bonding. J Med Chem 57:6403–6418. doi:10.1021/jm500697c
187. Singh S, Mandlik V (2015) Structure based investigation on the binding interaction of transport proteins in leishmaniasis: insights from molecular simulation. Mol Biol Syst 11:1251–1259. doi:10.1039/c4mb00713a
188. Tomkiewicz D, Casadei G, Larkins-Ford J, Moy TI, Garner J, Bremner JB, Ausubel FM, Lewis K et al (2010) Berberine-INF55 (5-nitro-2-phenylindole) hybrid antimicrobials: effects of varying the relative orientation of the berberine and INF55 components. Antimicrob Agents Chemother 54:3219–3224. doi:10.1128/AAC.01715-09