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REVIEW ARTICLE

Structural Biology Meets Drug Resistance: An Overview on Multidrug Resistance Transporters

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Abstract | Structural biology provides snapshots of biological function of molecular- and atomic-level structures of macromolecules, and holds great promise in addressing the emerging problems of biomedical science. Since the discovery of penicillin in early twentieth century, mankind has become aware and confronted with the emergence of antibiotic-resistant pathogens. In parallel to the failure of antibiotic therapy against infectious pathogens, there had been continuous reports of cancerous cells not responding to chemotherapy with increase in the duration of therapy. Research on the underlying causes of multidrug resistance in cancerous cells and later on in infectious bacteria revealed the involvement of integral membrane transporters, capable of recognizing a broad range of structurally different molecules as substrates and exporting them from the cell using cellular energy. Structural biologists succeeded in determining the structure of AcrB from *Escherichia coli* in 2002, the first structure of a multidrug resistance (MDR) transporter, and since then rapid progress has been made in the structural elucidation of these transporters. To date, structures of these transporters in *apo*- and substrate/inhibitor-bound state have been determined and deposited in the protein databank. This repository is a valuable source for structure-based drug design against MDR pumps. In this review, major findings related to structural biology of MDR transporters belonging to three major superfamilies, viz., ATP-binding cassette superfamily, major facilitator superfamily and resistance nodulation division superfamily are presented. Further, the future role of structural biology in improving our understanding of drug–transporter interactions and in designing novel inhibitors against MDR pump are discussed.

1 Introduction

Structural biology, since its inception in 1926, with the crystallization of urease enzyme by Sumner[1] has been assisting researchers in comprehending the molecular basis of life. Major techniques being used in this field include X-ray diffraction, electron microscopy (EM), especially cryo-EM and nuclear magnetic resonance (NMR) spectroscopy. X-ray fiber diffraction has been instrumental in the development of double-helix model of deoxyribonucleic acid[2] in finding the molecular basis of protein functions, such as lysozyme[3] and myoglobin,[4] complying with the motto of structural biology, i.e., "structure is function".[5] As of January 27, 2017, there are 117,184 known protein structures in the protein data bank (PDB; http://www.rcsb.org/).

Structural biology has a great potential in addressing the emerging problems of clinical science, such as the challenge posed by emergence

Cryo-EM: Cryo-electron microscopy (cryo-EM), is a form of transmission electron microscopy where the sample is analysed at cryogenic temperatures (generally liquid-nitrogen temperatures). This technique is gaining popularity in determining structure of proteins and large biomolecules.

X-ray fiber diffraction: A method used to determine the structural information of a molecule by using scattering data from X-rays.

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and reemergence of bacterial and viral infections, microbes with new resistance mechanisms, e.g., pandrug and multidrug resistance (MDR) in *Mycobacterium tuberculosis* and *Staphylococcus aureus* and worldwide increase in the incidence of recalcitrant cancers. Resistance against clinically prescribed antibiotics and cytotoxic drugs is a global health concern and its severity is increasing day by day coupled with the shortage of new active antibiotics and/or chemotherapies that can checkmate the disease. Well-known mechanisms primarily responsible for resistance phenotypes include alteration of the drug target, enzymatic inactivation/modification of the drug, changes in membrane permeability and enhanced antibiotic efflux.[6–8] Antibiotic efflux mediated by MDR transporters is an effective resistance mechanism with far-reaching consequences since several MDR transporters individually extrude clinically important drugs of diverse chemical structures out of the cell, and hence multiply the effect of other mechanisms.[9, 10] An in-depth understanding of the molecular basis of multiple substrate recognition by MDR transporters and translocation mechanisms is needed to design novel drugs and efflux pump inhibitors.

MDR transporters: Membrane proteins that extrude drug compounds out of the cell.

Currently, three-dimensional structures of several MDR efflux transporters have been determined primarily by X-ray crystallography. However, the gradual development of electron microscopy methods has enabled the visualization of biological sample in a closer-to-native state[11] and structural biologists have started unveiling the macromolecular organization in new dimensions, e.g., single-particle analysis of tripartite multidrug efflux transporter from *E. coli* (AcrA–AcrB–TolC) and *Pseudomonas aeruginosa* (MexA–MexB–OprM) by electron microscopy revealing the formation of a stable interspecies AcrA–MexB–TolC complex, which is suggestive of a common mechanism of tripartite assembly.[12]

Single particle analysis: Image processing techniques to build a three dimensional structure from transmission electron microscopy images, typically of proteins or large biomolecules such as viruses.

Multidrug efflux transporters are found among five transporter superfamilies, viz., ATP-binding cassette (ABC) superfamily,[13] major facilitator superfamily (MFS),[14] resistance nodulation division (RND) superfamily,[15] multidrug and toxic compound extrusion (MATE) superfamily,[16] and small multidrug resistance (SMR) superfamily.[17] In this review, we will describe the major structural findings of multidrug transporter belonging to ABC superfamily, MFS, and RND superfamily. Exporters from ABC superfamily play a prominent role in resistance against cancer, whereas members of MFS and RND are significant with reference to infectious bacteria. Finally, we will also discuss the future aspects of research aimed at tackling MDR pump inhibition.

2 P-gp, P-glycoprotein—A Therapeutic Target Among Multidrug-Resistant ABC Transporter

A typical ABC transporter, whether importer or exporter, comprises a minimum functional unit of four domains: two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) (Fig. 1), collectively made up by two polypeptide chains. Since structures of NBDs are conserved in all the ABC transporters, based on known structural architecture of TMDs, the ABC transporters fall into three groups: type I ABC importers, such as ModBC and MalFGK2; type II ABC importers such as BtuCD and type III ABC exporters including multidrug efflux transporters, such as Sav1866, and p-glycoprotein.[18]

ABC multidrug efflux transporters are present in both prokaryotes and eukaryotes. The first structure of a complete ABC multidrug efflux transporter was reported for Sav1866 from *Staphylococcus aureus* by X-ray crystallography;[19] another structure, that of BmrA from *Bacillus subtilis*, was determined by cryo-EM.[20] However, the breakthrough in cancer research started with the structural elucidation of P-glycoprotein (P-gp),[21] also known as ABCB1. Apart from clearance of xenobiotics from normal cells using ATP as the source of energy of transport cycle,[22] P-gp is also responsible for conferring an MDR phenotype on tumor cells. P-gp is a validated therapeutic target and, so far its three-dimensional structures have been determined from *Mus musculus* (mouse),[21–24] *Cyanidioschyzon merolae* (red alga)[25] and *Caenorhabditis elegans* (nematode)[26] (Fig. 2).

Since human P-gp is 87% identical to mouse P-gp,[24] and 46% to *C. elegans* P-gp,[26] the findings from these structures can help understand the functionality and mechanism of transport by human P-gp. From the crystal structure of mouse P-gp, it was found that amino acid residues occupying the drug transport pathway are 96% identical to human P-gp, with only two non-identical residues between mouse and human P-gp.[24] The substrate translocation pathway was found to have nine conserved aromatic residues, which are assumed to play a predominant role in polyspecific substrate recognition.[24] The residues lining the drug-binding pocket of mouse P-gp provide evidence for electrostatic drug–protein interaction including cation–ϖ, CH–ϖ or ϖ–ϖ interactions.[24] The three-dimensional structure of P-gp

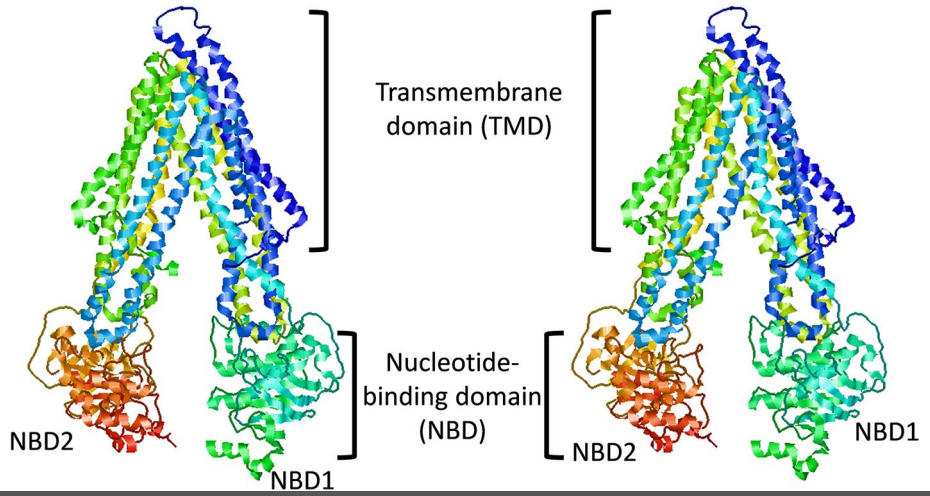

Figure 1: Stereo view of P-gp structure in inward-open conformation; nucleotide-binding domain (NBD) is the site for the hydrolysis of ATP, while transmembrane domain (TMD) comprises transmembrane helices. Each ABC transporter functions in dimer, with each monomer comprising an NBD and TMD. Adapted from Ref. [24]

in *apo*-form revealed a spacious internal cavity, occupying a volume of ~6000 Å^3; this large polyspecific drug-binding pocket of P-gp recognizes a variety of chemotherapeutic compounds. While effluxing these therapeutics across the membrane, P-gp undergoes changes in the size and shape of drug-binding pocket, until the drug is thrown out of the cell.[21] It was also observed that the drug-binding pocket of mouse P-gp was stereo-selective. [21], [24] The gateways for uptake of substrate open into the cytoplasm and the lipophilic interior of membrane bilayer[21] with transmembrane helices IV and VI together form intra-membranous drug entry portal to the binding pocket.[23] In the structure of *C. merolae* P-gp, whose amino acid composition, multidrug specificity, and kinetics of ATP hydrolysis are very similar to human P-gp,[25] it has been proposed that substrates enter from the inner leaflet of the lipid bilayer, where transmembrane helix IV acts as gatekeeper, and the flexibility of helix IV allows uptake of substrates of various sizes and structures. The exit gate of *C. merolae* P-gp consists of four-helix bundle of transmembrane helices I and VI from each TMD, along with transmembrane helix V of each domain. Tyrosine residue (Tyr358 in CmABCB1) has been proposed to induce the opening of the extracellular exit gate and acceleration of ATPase activity.[25] According to the transport mechanism proposed for ABC efflux transporters, the transporter in an inward-facing conformation binds substrate from the cytoplasm or the inner leaflet of the membrane bilayer. Subsequently, binding of two molecules of ATP dimerizes NBDs and switches the TMDs from the inward-facing to an outward-facing conformation, resulting in the extrusion of the substrate. ATP hydrolysis dissociates the NBDs and resets the transporter to the inward-facing conformation[27], [28] (Fig. 3).

Mouse P-gp structures co-crystallized with inhibitors have established that the P-gp can be inhibited by blocking dimerization of NBDs, which is essential for ATP hydrolysis,[22] and by blocking the substrate-binding pocket of TMDs.[29] Hence, rational design of inhibitors can be carried out to defeat recalcitrant tumor cells. These inhibitors can be prescribed along with the anti-tumor drug to block the transporter for effective drug treatment. However, their prescription depends upon the resistance pattern of tumor cells.

Stereo-selective: Only one stereoisomer among a mixture of stereoisomers reacts with the target molecule.

3 MdfA, Multidrug Facilitator A—A Paradigm of Multidrug Extrusion Transporter Belonging to Major Facilitator Superfamily

The major facilitator superfamily (MFS) is the largest superfamily of secondary active transporters comprising over 15,000 sequenced members divided into 74 families and found in all kingdoms of life.[30] Infectious bacteria have evolved multidrug efflux pumps of the MFS that can extrude many structurally unrelated antibiotics by utilizing the H^+ electrochemical gradient as source of energy.[31] To date, three MFS MDR pumps, all from *E. coli*, have been described

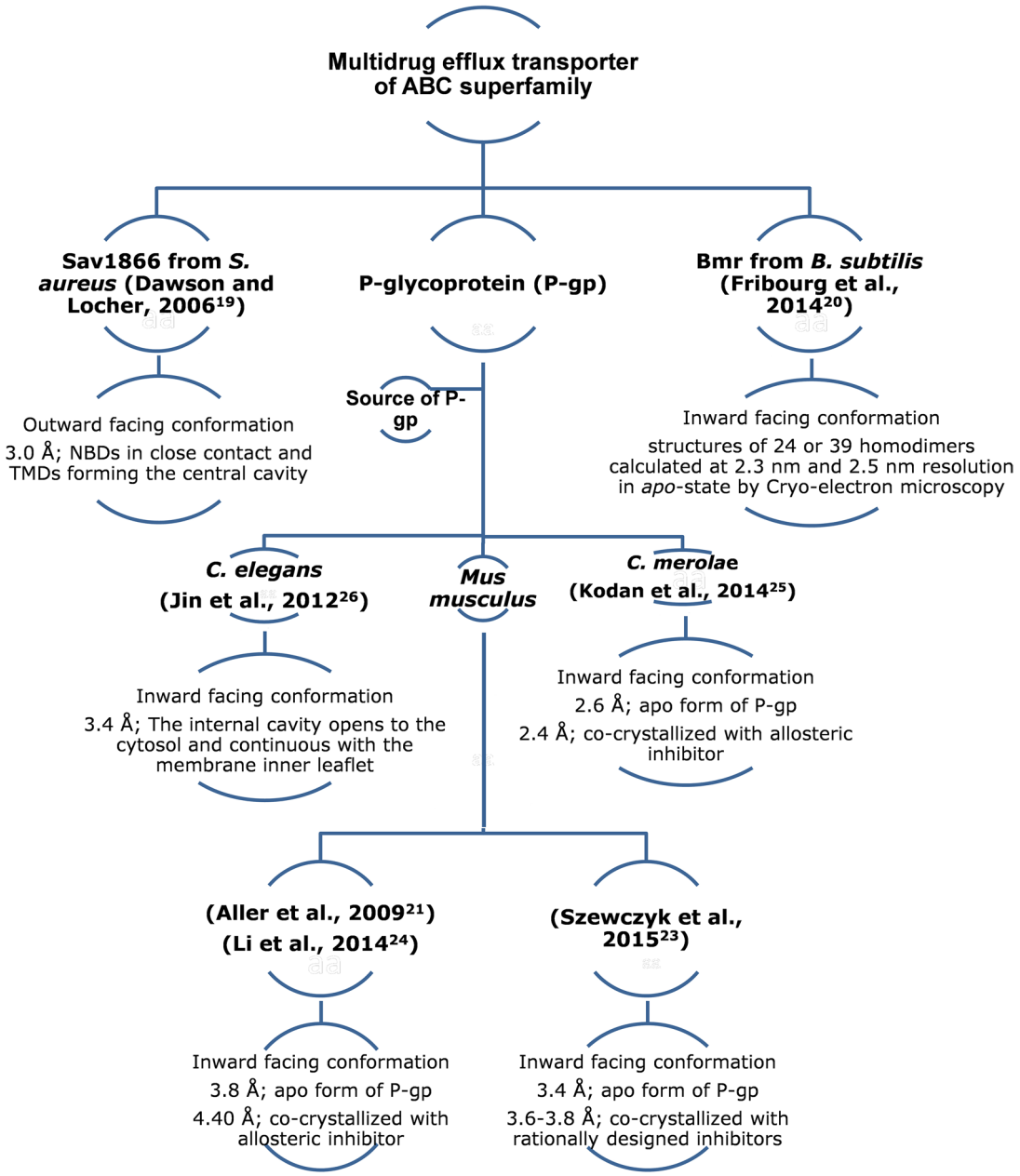

Figure 2: Summary of ABC P-gp efflux transporters with known structures. ***NBD*** nucleotide-binding domain, ***TMD*** transmembrane domain.

structurally: EmrD,[32] YajR[33] and MdfA[31]. EmrD and YajR have not yet been characterized thoroughly in terms of their biochemical and functional properties. MdfA on the other hand has been studied extensively and serves as paradigm for MFS MDR pumps.

The MFS transporters, including EmrD, YajR and MdfA, exhibit a core architecture of 12 transmembrane α-helices, with distinct N- and C-terminal domains,[31] more recently an MFS fold, made up of a "3-helix structure" repeat has been described as MFS transporter.[34] α-helices I to VI in the N-terminal part form N-domain and α-helices VII–XII in the C-terminal part form C-domain. Both N- and C-domains are connected by interdomain, amphipathic helix (Fig. 4). Transmembrane α-helices forming the central cavity include helices I, IV, VII and X,[31] whereas remaining helices assist in conformational changes during the transport cycle and are referred to as rocker helices and support helices. The amino acid residues lining the central cavity of MDR pumps from the

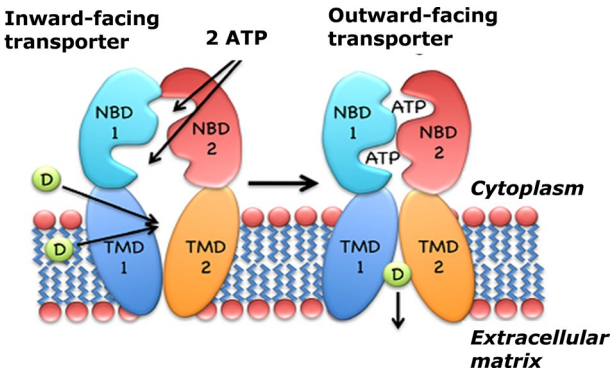

Figure 3: Scheme of drug transport by ABC efflux transporter, adapted from Ref. [27]

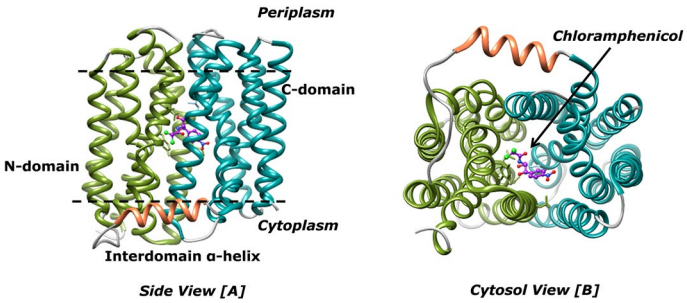

Figure 4: Structural biology of a typical MFS transporter, i.e., MdfA, co-crystallized with chloramphenicol (PDB ID:4zow). **a** Viewing parallel to membrane, **b** viewing from the top. N- and C-domains are represented in *green* and *cyan*, respectively. Inter-domain α-helix is shown in *orange*. Structural figures of MdFA have been adapted from Ref. [31] with permission.

MFS are predominantly hydrophobic. MdfA co-crystallized with chloramphenicol shows central cavity of ~3000 Å^3 in size. Since, among the substrates of MdfA include neutral antimicrobials, such as, chloramphenicol and mono-cationic substrate like triphenylphosphonium (TPP^+), and one proton is transported per efflux cycle, the extrusion of chloramphenicol by MdfA is electrogenic, while that of TPP^+ is electroneutral. [31]

The MFS are characterized by four consensus motifs, all of which are present in the crystal structure of MdfA.[31] Motif A has been postulated to stabilize the outward-facing conformation of MFS transporters.[33] Motif B in transmembrane α-helix IV contains Arg, a basic residue; the positive electrostatic field generated by Arg112 in inward-open state of MdfA favors deprotonation of Asp34.[31] Motif C, also called anitporter motif, in the transmembrane α-helix V stabilizes the inter-domain interaction of MFS antiporters in the inward-open state via hydrophobic interaction. Motif D has two proton-titratable amino acid residues, Glu26 and Asp34. According to chloramphenicol-bound crystal structure of MdfA, Asp34 is directly involved in substrate binding and it might induce deprotonation of Asp34 and therefore is considered a major protonation site in inward-facing conformation of transporter. As translocation cycle proceeds, Glu26 becomes the major protonation site in the outward-facing conformation.[31]

A mechanism for the transport cycle has been proposed based on biochemical and structural data. In the inward-facing conformation of transporter, upon substrate binding Asp34 undergoes deprotonation, which then triggers the inward-to-outward conformational change. In the outward-facing conformation, releasing of the substrate induces protonation of Glu26. This protonation and negative inside rule for membrane potential induces the conformational switch from outward to inward state. In short, a transport cycle is driven by protonation of the transporter and the membrane potential[31] (Fig. 5).

The detailed characterization and significance of MFS motif residues in substrate binding, domain stabilization and translocation mechanism suggest that these motifs may be well-suited

Electrogenic: A transport process, for example, that leads to the translocation of net charge across the membrane.

Electroneutral: A particle or body or system having no net electric charge as a result of some process.

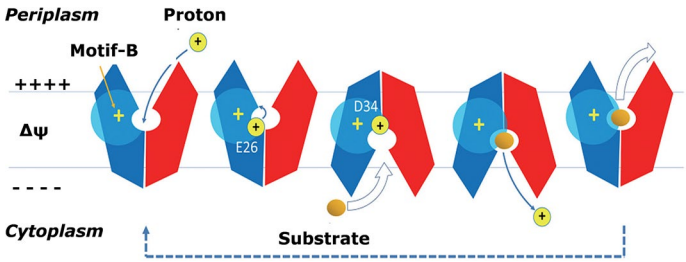

Figure 5: Scheme of transport by MdfA, figure has been adapted from Ref. [31] with permission.

targets for rational design of inhibitors of MFS MDR pumps.[35]

4 AcrB, Acriflavine Resistance Protein B: A Prototype of Tripartite Multidrug Efflux Transporter from Resistance Nodulation Division Superfamily

Resistance nodulation division (RND) superfamily consists of tripartite efflux system, found exclusively in Gram-negative bacteria, including genus from *Salmonella enterica* and *Pseudomonas aeruginosa*. The basic architecture of RND transporter comprises inner membrane RND component, periplasmic adapter protein and outer membrane channel. RND transporters with structurally known biology include MexB from *P. aeruginosa* and AcrB from *E. coli*. AcrB is particularly well characterized in terms of structure with more than 50 entries in PDB (http://www.rcsb.org/) (Fig. 6), including the *apo*-form,[36] substrate-bound form with low-molecular mass antibiotics such as ciprofloxacin and nafcillin[37, 38] and high-molecular mass antibiotics, such as rifampicin and erythromycin.[39, 40] AcrB bound with an analogue of its natural substrate, i.e., bile acid, has also been reported.[41] DARPin

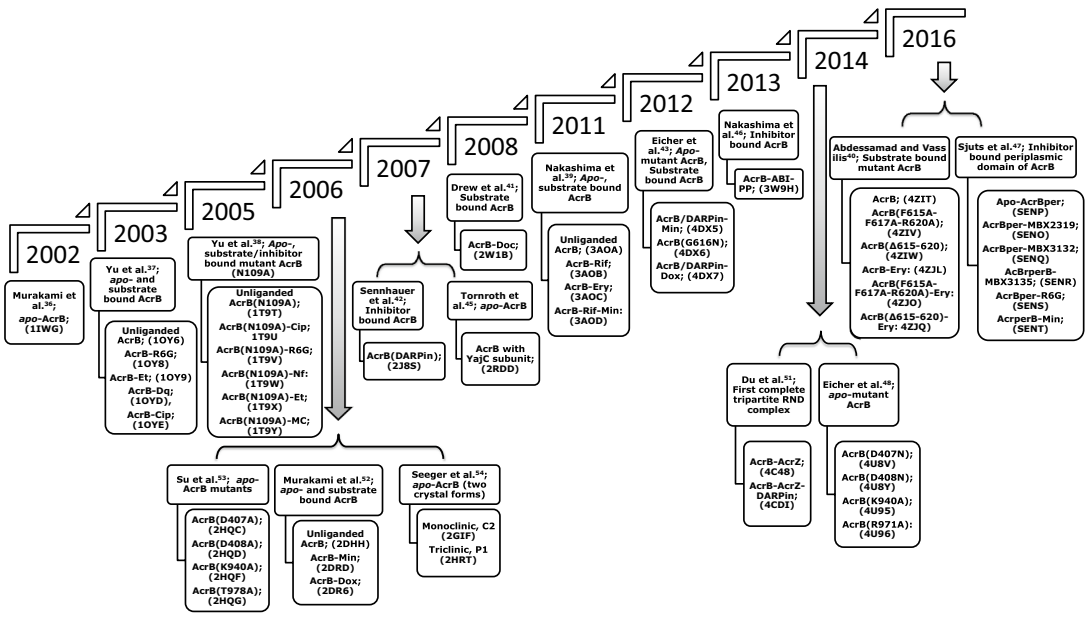

Figure 6: Year-wise development in the structure determination of AcrB; PDB identifiers have been given *within brackets* (). Among substrates co-crystallized with AcrB include various antibiotics and dyes, where Et ethidium bromide; R6G rhodamine 6G; Dq dequalinium; Cip ciprofloxacin; Nf nafcillin; MC Phe-Arg β-naphthylamide; Min minocycline; Dox doxorubicin; Ery erythromycin; Rif rifampicin; Doc deoxycholate; ABI-PP *tert*-butyl thiazolyl amino carboxyl pyridopyrimidine; DARPin designed ankyrin repeat proteins inhibitor; AcrBper a soluble version of AcrB, i.e., AcrB periplasmic domain. Genetically engineered AcrB have also been crystallized including point mutants N109A, D407A, D408A, K940A, T978A, G616N, D407N, D408N, K971A; a triple AcrB mutant (F615A–F617A–R620A); and deletion mutant Δ615–Δ620.

(designed ankyrin repeat protein inhibitor)-bound AcrB structures have been reported,[42, 43] whereas DARPins are small, single-domain proteins (14 kDa), which are promising target for the next generation of protein therapeutics.[44] Other structures of AcrB include AcrB in complex with YajC subunit,[45] AcrB in complex with pyridopyrimidine[46] and pyranopyridine inhibitors[47] and functionally inactive AcrB variants.[48] It is worth mentioning that although individual structures of TolC, AcrB and AcrA proteins were reported in 2000,[49] 2002,[36] and 2006,[50] respectively, the first structure of complete tripartite assembly (AcrA–AcrB–TolC from *E. coli*) was published by Du et al.[51] as recently as in 2014.

Each protomer of the AcrB trimer is composed of one transmembrane domain and two large periplasmic domains, namely, porter domain and TolC-docking domain (Fig. 7).[52] The trimeric architecture of AcrB is stabilized and maintained by interactions between loops from adjacent protomers. The transmembrane domain comprises 12 transmembrane α-helices, in which transmembrane helices-IV and -X are in the center surrounded by 10 transmembrane α-helices (TMH). Periplasmic domains are

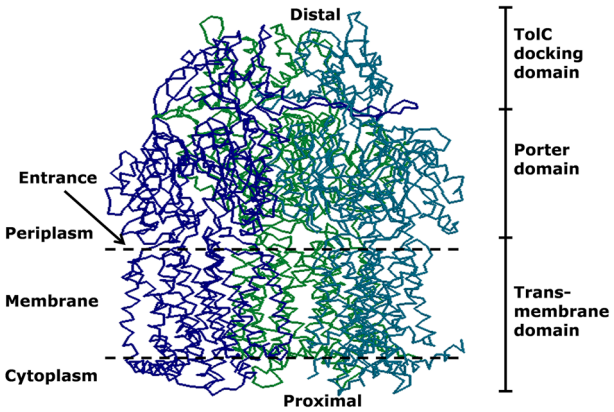

Figure 7: Architecture of trimeric AcrB transporter showing three domains of RND transporter with entrance site for drug in porter domain. Ribbon representation viewed from the side parallel to the membrane plane. The extra-membrane (periplasmic) headpiece is at the ***top*** and the transmembrane region is at the ***bottom***. Adapted from Ref. [52].

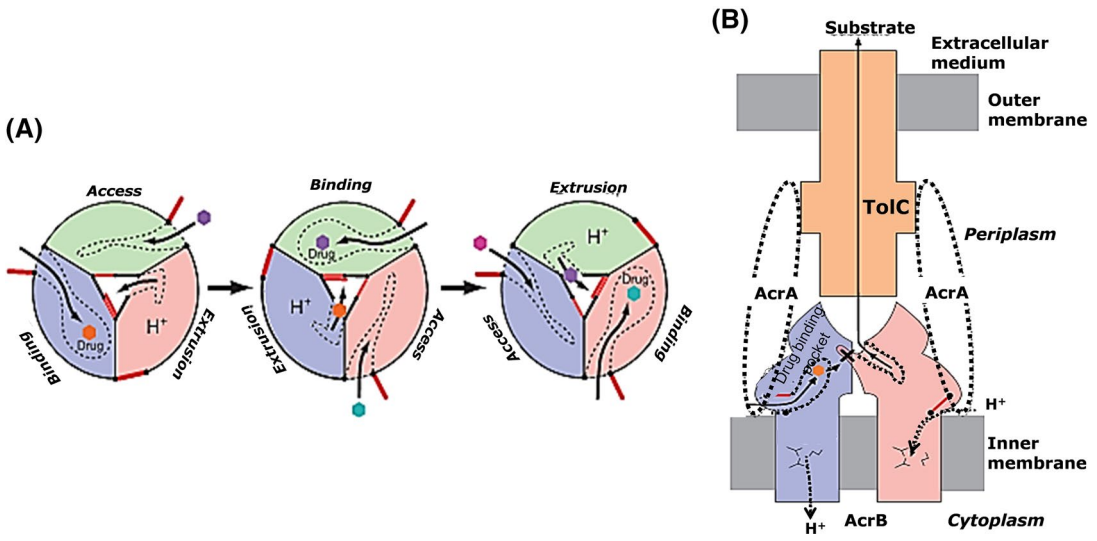

Figure 8: Illustration of the substrate transport mechanism proposed for AcrB. **a** Top view from the distal side of the cell. **b** Side view of tripartite complex of the drug entrance and exit sites within each monomer have been depicted as red flaps. The drug-binding pocket and translocation pathway are represented by dotted lines. Hexagons represent drugs. Adapted from Ref. [52]

formed by the folding of two connecting loops between TMH-I and TMH-II, and TMH-VII and TMH-VIII. Porter domain, which is next to transmembrane domain in AcrB architecture comprises four subdomains, i.e., PC1, PN1, PC2 and PN2. PC1 and PC2 subdomains accommodate adapter protein of tripartite complex, viz., AcrA in case of AcrA–AcrB–TolC complex,[52] whereas PN1 subdomains comprising three α-helices from each monomer together form a central pore of trimeric AcrB. TolC-docking domain further comprises DN and DC subdomains, and is located away from the membrane plane. A funnel-like structure of TolC domains narrows down towards porter domain.[52]

The structure of AcrB is available with symmetric monomers as well as with asymmetric monomers.[36, 37, 52, 53] In the latter, the individual monomer depicts a specific stage of transport cycle, namely, access, binding and extrusion mediated by L (loose), T (tight) and O (opens) monomers;[54] the T monomer shows a substrate-binding pocket inside the porter domain, lined by hydrophobic residues, which is not present in L and O monomers. From the asymmetric structures, a model for drug transport based on conformational cycling of the monomers has been proposed (Fig. 8). As per this model, the asymmetric monomers can assume any of the conformational states, L, T, and O, and transport is dependent on the concerted cycling of monomers through these three states and back to L state. L conformation substrates access state from periplasm and/or membrane for translocation. During the L to T transition, substrate moves toward the deep substrate-binding pocket inside the porter domain. Upon conformational change toward the O state, substrate is expelled from the binding pocket and exits AcrB transporter for final expulsion by TolC tunnel. During a complete cycle of access, binding and release of substrate, occlusions and constrictions inside the porter domain drives the unidirectional transport of substrate. This unidirectional movement involves subdomain movements. Hence, this was named as the "peristaltic pump mechanism".[39]

The ciprofloxacin-, dequalinium-, rhodamine 6G- and ethidium-bound structures of AcrB showed that three molecules of each ligand bind simultaneously to the extremely large central cavity of 5000 $Å^3$ and their interaction stabilizes the binding.[37] Another study, carried out on AcrB mutant (Asn109Ala), co-crystallized with five structurally diverse ligands—ethidium, rhodamine 6G, ciprofloxacin, nafcillin, and Phe-Arg-naphthylamide—a substrate-binding site was proposed in periplasmic domain formed by the C-terminal periplasmic loop.[38] Yet in another study, carried out on AcrB co-crystallized with high-molecular mass drugs, i.e., rifampicin and erythromycin with asymmetric conformation of AcrB monomers, it was proposed that high-molecular mass drugs first bind to the proximal drug-binding pocket of L monomer of AcrB, and are then forced into the distal binding pocket of T monomer of AcrB by a peristaltic mechanism described earlier. For low-molecular mass drugs, such as minocycline and doxorubicin, a relatively different transport mechanism has been suggested, which involves transfer of drugs through proximal binding pocket without specific binding until they reach distal binding pocket, which specifically binds these low-molecular mass drugs.[39] Involvement of two discrete, spacious and multisite-binding pockets forms the basis for the extraordinary broad substrate profile of AcrB.

5 Future Directions

So far significant efforts have been made to understand the molecular mechanisms underlying drug transport by studying *apo*- and substrate-bound structures of MDR efflux pumps. However, owing to the complex architecture of efflux transporters, especially those belonging to RND and ABC superfamilies, such efforts will go a long way to design structure-based drugs.

In the case of P-gp, only the structure of an inward-facing conformation is available; to validate the proposed transporter cycle for ABC exporters, additional structures with outward-open and occluded conformations are needed. Moreover, it has been known that two ATPs are hydrolyzed per transport cycle of ABC efflux transporters; however, no structural verification exists for the mechanism of ATP hydrolysis. Substrate competition and substrate preference for this exceptionally broad-spectrum transporter need structural evidence with co-crystallization of the transporter with multiple substrates. Similar is the case with MFS MDR transporters, where MdfA from *E. coli* is available in only one inward-open conformation and determination of intermediate conformations of transporter cycle will further illuminate the transporter mechanisms and substrate recognition/binding. AcrB has been structurally characterized in multiple conformations. Various structures available with rationally designed inhibitors for AcrB and pyranopyridine derivatives have been reported to increase the potency of several antibiotics, especially of levofloxacin and piperacillin against *E. coli* and

other Enterobacteriaceae.[47] However, since this structure is available for an engineered version of AcrB, wild-type AcrB, when co-crystallized these inhibitors will mimic inhibition by these inhibitors in closer to native state.

So, overall the following points need attention in the future for structural elucidation of efflux transporters discussed in this review:

- Capturing of *apo*-structure of P-gp with intermediate states of transport cycle.
- Substrate-bound P-gp structure determination.
- *apo*-MdfA structure determination.
- Positively charged substrate-bound MdfA structure determination.
- Substrate (chloramphenicol)-bound structure of MdfA with intermediate states of transport cycle, viz., outward-open and occluded state.
- Wild-type AcrB structure determination in complex with pyranopyridine inhibitors.

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