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SUPPLEMENTARY INFORMATION

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Multidrug efflux pumps: structure, function and regulation

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Supplementary Box 1 | **Drug efflux pump inhibition and other avenues for antimicrobial drug design**

Active drug efflux, especially that caused by the RND pumps, has a major role in intrinsic and enhanced resistance in Gram-negative bacteria. Many antibiotics are substrates for active efflux and are rendered ineffective owing to overexpression of pumps in pathogens. The broad substrate range of the major multidrug exporters present a challenge for antibiotic development against all bacteria, but this challenge is even greater in the case of the Gram-negative bacteria owing to the need for the drug to traverse both the inner and outer membranes, which represent a formidable permeability barrier. In principal, small, hydrophilic drugs could target multidrug efflux pumps, but these do not readily enter the periplasm. Countering the action of pumps by RND pump inhibitors may be efficacious in adjuvant therapy. For example, RND pump inhibition improved the activity of a FtsZ-directed anti-staphylococcal prodrug against *E. coli*, *K. pneumoniae* and *A. baumannii* [1].

Previous efforts to design efflux pump inhibitors have targeted the inner membrane section of the pumps or the core of the outer membrane conduit [2] . Their use in bacteria has demonstrated that inhibition of efflux pumps can reverse multidrug resistant phenotypes and render them highly susceptible to common antibiotics. In combination with conventional antibiotics such inhibitors may broaden the activity of Gram-positive antibiotics to the Gram-negative species, which currently account for 80% of clinical MDR infections. The dipeptide amide PAβN (Phe_arg-beta-napthylamide) and the aryl piperazine NMP are capable of partially restoring the activity of many antibiotics including levofloxacin, chloramphenicol, and erythromycin in drug-resistant strains of Gram-negative bacteria [3,4]. In drug-resistant *E. coli* and *P. aeruginosa*, the competitive inhibitors D13-9001 (a pyridopyrimidine compound) and MBX2319 (a pyranopyridine derivative) have been reported to return partial efficacy to a wide array of antibiotics. To date, the only known inhibitors that bind tightly to the distal site in a deep binding pocket (as shown by co-crystal structures) and block efflux are the pyranopyridine derivatives MBX 2319, MBX2931, MBX3132, and MBX3135 [5] and the pyridopyrimidine compound D13-9001[6]. The crystal structure of *E. coli* AcrB in complex with D13-9001 reveals that the hydrophobic portion is bound in a 'hydrophobic trap' in the distal binding pocket and is surrounded by F136, F178, F610, F615, and F628, while the more hydrophilic moieties are bound to the upper, groove-like portion of the binding pocket [6]. This structure suggests that this particular inhibitor prevents the binding of other substrates to the upper part of the binding pocket. The complex of AcrAB-TolC bound to MBX3132 has recently been structurally characterized by cryoEM and shows that the ligand can bind to all three pockets in AcrB in the 'tight' binding state, that is, AcrB displays a TTT conformation [7], whereas efflux substrates bind to only one tight conformational state at a time, that is, AcrB displays a LTO conformation. The inhibitor binding is inferred to trigger conformational changes that are transmitted across the entire assembly to open the TolC channel from a closed state.

It is of interest that inhibitors of the pump form defined molecular interactions and resemble the typical high affinity ligand-receptor binding interactions, including van der Waals interactions with the hydrophobic side chains, filling the available space, and hydrogen bonding patterns, sometimes including water molecules [5,6]. Clearly these more defined interactions increase the binding energy, to an extent that even the conformational transitions of the pump might be impeded by the bound inhibitor. Moreover, a deeper portion of the distal binding pocket, known as the 'hydrophobic trap' contains conserved Phe residues and is where the hydrophobic part of the inhibitors as well as some substrates make extensive contact [5,6,8]. It has been proposed that loose interaction with the hydrophobic trap may enhance the efflux of substrates, but too tight binding could impede efflux of compounds [8,9]. The inhibitor PAβN, which inhibits the efflux of other drugs by binding partly to the hydrophobic trap and also by sterically occluding the binding of other drug substrates to the upper part of the distal pocket, is also a transported substrate of AcrB. It is perhaps unexpected that the binding of the inhibitor is weaker than that of some known substrates [9,10]. The capacity of aminoacyl beta-naphthylamides to act as inhibitors or activators depends on how they bind to the distal pocket and engage the hydrophobic trap [5,6,9]. Another consideration in rationalizing ligand binding strengths versus transport rates is that the process is out of equilibrium when driven, and is likely to involve processes such as rates of changes to substrate hydration that can occur during conformational switching.

One of the difficulties faced in rational targeting of bacterial machinery is to find compounds that can enter the cell efficiently. A set of experimentally based rules has been elaborated for the influx of compounds through porins [11]. These rules could be useful to define strategy for compounds to enter the periplasm of Gram-negative bacteria to target tripartite assemblies or the periplasmic domains of the stand-alone

transporters. The compounds reported that inhibit RND transporters are a promising strategy for this approach. The open conduit of TolC can be another path of access that can greatly widen the chemical space for inhibitors. For example, potential inhibitors that act as channel blockers might include polar compounds that bind avidly to the electronegative surface of the constriction point of TolC observed in the resting state of the isolated protein and in the apo form of the assembled pump [7]. Apart from being used as potentiators of antibiotics, efflux inhibitors can also be used as potential anti-virulence drug since inhibition of efflux attenuates virulence [12].

There is growing appreciation for how community behavior of complex microbiota has an impact on the dynamic interactions, with potential impact on host virulence. Selective treatments that target the infecting bacterium but do not impact on the composition of the microbiota are ideal. Inhibition of biofilm formation or the adhesion of bacteria to specific sites in the host (e.g. type 1 pili interactions with epithelial cells in uropathogenic *E. coli* [13]) might be a suitable target. This might be achieved by inhibitors that target these processes, including tripartite assemblies that secrete the substances to form biofilms. The relationship between drug exporters and biofilm formation varies in different species. The loss or inhibition of any of 9 MDR pumps (AcrAB, AcrD, AcrEF, MdtABC, MdsABC, EmrAB, MdfA, MdtK, and MacAB) or the TolC OM protein in *Salmonella enterica* impairs biofilm formation with reduced production of the surface protein curli [14]. However, *P. aeruginosa* mutants can form biofilms without requirement for MDR pumps [15]. KpnEF, an SMR pump, contributes to MDR and may be important in the infection process, because its expression is regulated by the Cpx regulatory system, which also affects capsule production [16].

Anti-sense nucleic acids may also be an avenue for manipulating genes related to the MDR phenotype. The CmeABC pump contributes to MDR in *Campylobacter jejuni*, and anti-CmeA and anti-CmeB peptide nucleic acids sensitize the bacterium to multiple antimicrobials [17]. Agents that increase membrane-permeability or counter suppression of porin biogenesis may also be avenues that increase drug susceptibility. The porins are often suppressed by small regulatory RNAs, and these could be countered by anti-sense nucleic acids.

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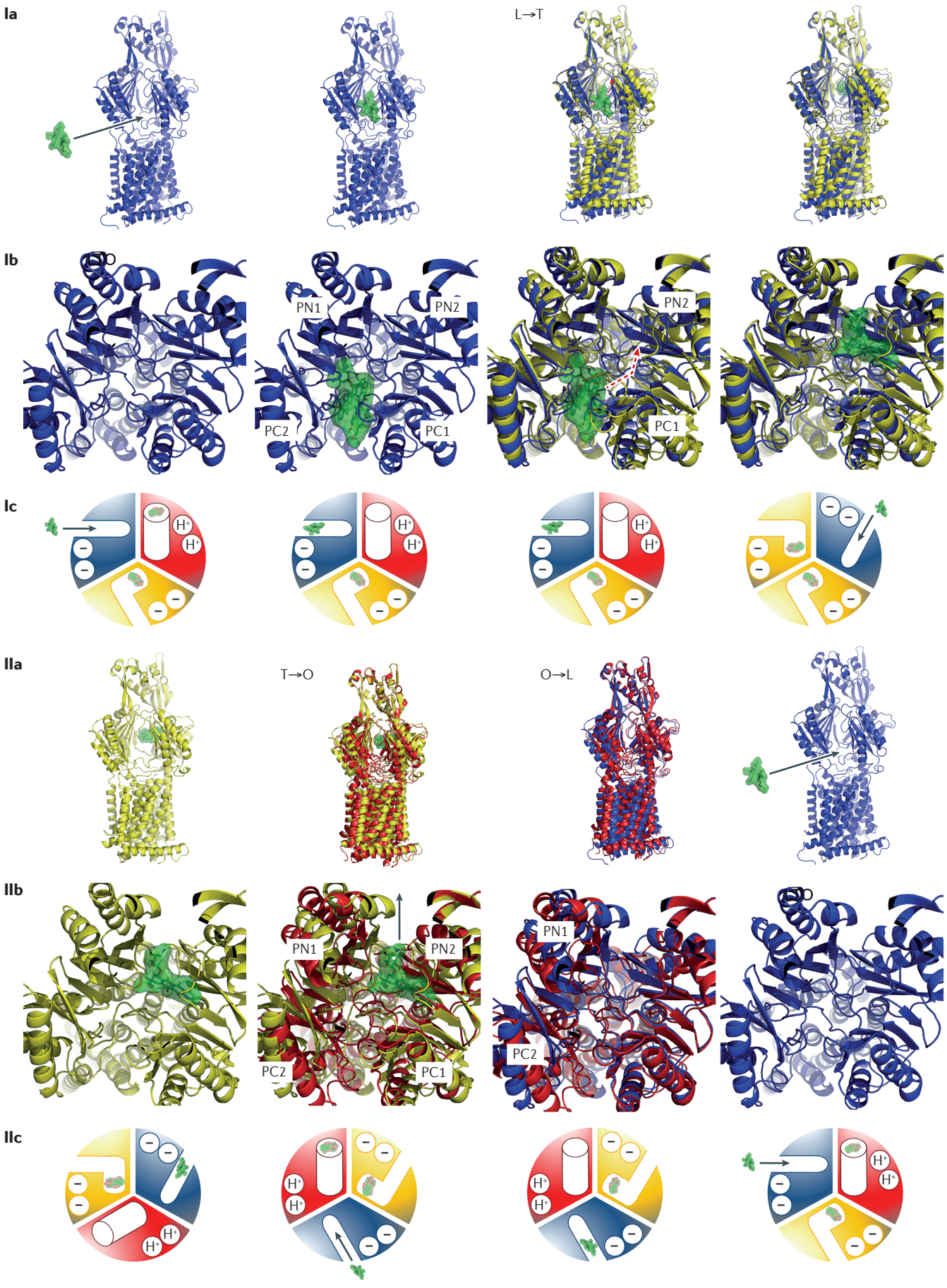
Ia
L→T
Ib
PN1
PN2
PC2
PC1
Ic
H+
IIa
T→O
O→L
IIb
IIc

Supplementary Figure 1 | **Conformational switching and substrate pathways of the RND transporter, AcrB**

Functional rotation of the single AcrB protomer and subdomain movement during the putative transport of doxorubicin. Panels Ia and IIa: side view of the AcrB protomer; Panels Ib and IIb: Periplasmic top view on the PN1, PN2, PC1, and PC2 subdomains of the periplasmic part of AcrB; Panels Ic and IIc: Wheel presentation of the functional rotation cycle and drug binding/transport/extrusion and proposed protonation status of the catalytically relevant Asp residues on transmembrane helix 4. From left to right: Doxorubicin (green, stick and surface representation, doxorubicin dimer) sequestering in the proximal binding pocket of the L protomer (cartoon representation, blue), delineated by the PC1/PC2 subdomains. L to T transition and movement of doxorubicin (monomer) into the distal binding pocket of the T protomer (yellow). The L protomer (blue) and T protomer (yellow) are shown in superimposition to indicate the movement of the PN2 and PC1 subdomains resulting in the formation of the distal binding pocket, and subsequent movement of doxorubicin into the distal binding pocket. Stabilization of the T conformation by drug binding induces the uptake of protons into the transmembrane domain and subsequent protonation of the two Asp residues, inducing the T to O transition. The latter transition, resulting in the release of the drug, is postulated to be the energy-dependent step in the cycle. The T protomer (yellow) and O protomer (red) are superimposed to indicate the concerted movement of all four subdomains, resulting in the closure of the proximal and distal binding pockets. The drug is squeezed out the distal binding pocket and moves through a T to O transition-induced exit tunnel towards the (AcrA) docking domain into the AcrB funnel. Release of the drug and the concerted movement of all four subdomains is transduced by conformational change to the transmembrane domain and results in the deprotonation of the Asp residues and release of the protons to the cytoplasm. Loss of these protons cause the O (protomer in red) to L (protomer in blue) transition. The model depicts two protons per cycle, but this is hypothetical, and a scenario with one proton per cycle is also feasible.