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Bacterial efflux transporters' polyspecificity - a gift and a curse?

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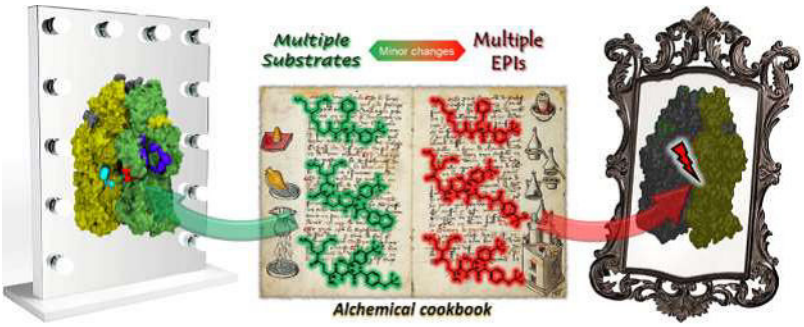
Abstract

All mechanisms of clinical antibiotic resistance benefit from activities of polyspecific efflux pumps acting to reduce intracellular accumulation of toxins and antibiotics. In Gram-negative bacteria, the major polyspecific efflux transporters belong to the Resistance-Nodulation-cell Division (RND) superfamily of proteins, which are capable of expelling thousands of structurally diverse compounds. Recent structural and functional advances generated novel insights into mechanisms underlying the biochemical versatility of RND transporters. This opinion article reviews these mechanisms and discusses implications of the polyspecificity of RND transporters for bacterial survival and for the development of efflux pump inhibitors effective in clinics.

Graphical Abstract



Keywords

antibiotic resistance; multidrug efflux; efflux pump inhibitors; polyspecific molecular transport

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Introduction

Bacteria adopt numerous and complex antibiotic resistance mechanisms, both intrinsic and acquired. Amongst these mechanisms, extrusion of antibiotics through efflux transporters is extremely effective. When first discovered in bacteria more than 25 years ago, multidrug efflux transporters were accentuated for their striking substrate polyspecificity [1,2]. Unlike typical poly-specific enzymes that tolerate minor modifications around chemical cores of compounds, polyspecificity of multidrug transporters encompassed substrates with various chemical scaffolds and physico-chemical properties. There is a large body of evidence that polyspecific efflux bestows multiple benefits for bacterial growth and proliferation. Polyspecific transporters are implicated in various aspects of bacterial physiology that require proliferation and spread into new environments including pathogenicity and virulence [3–6], cell-to-cell communication [7,8], biofilm formation and the efflux of secondary metabolites and toxic intermediates [9,10]. In clinics, polyspecificity of efflux pumps is the major driver for mutations needed to gain antibiotic resistance [11]. Polyspecificity also enables redundancy. A typical bacterial cell possesses several structurally and regulatory unrelated transporters able to remove the same molecule [12]. As a result, most genes that encode efflux pumps appear to be non-essential for bacterial growth [13]. For example, *E. coli* strains lacking up to ten various efflux pumps as well as *P. aeruginosa* lacking up to seven major efflux pumps do not have profound growth defects under laboratory conditions [14–17].

Among the different families of bacterial efflux transporters, members of the Resistance-Nodulation-cell Division (RND) superfamily (Fig. 1) are the dominant efflux power of Gram-negative bacteria and represent a paradigmatic example of polyspecificity [18]. In this opinion article, we will focus only on RND transporters, highlighting recent advances in understanding mechanisms underlying polyspecificity of these transporters and emerging efflux inhibition strategies.

Efflux pumps of the RND superfamily: a paradigm of poly-specific transport

RND transporters can expel from cells an extremely broad range of compounds. Synergistic interactions with the low permeability barrier of the outer membrane (OM) are one of the enabling mechanisms of such polyspecificity. RND pumps functioning in the context of two membranes appear to be powerful with slowly permeating compounds even though such compounds are very poor substrates in biochemical terms [15,19,20]. Most RND efflux pumps function as tripartite complexes comprised of 1) an inner membrane (IM) RND transporter, 2) a periplasmic membrane fusion protein (MFP), and 3) an OM factor (OMF, Fig. 1). Notorious examples of these complex biological assemblies are AcrAB-TolC and MexAB-OprM, from *Escherichia coli* and *Pseudomonas aeruginosa*, respectively, featuring an IM transporter (AcrB/MexB), a MFP (AcrA/MexA), and an OMF (TolC/OprM) with stoichiometry 3:6:3 [21,22].

The IM protein plays a central role in the activity of the whole efflux assembly, being responsible for recognition and capture of compounds from within the cell and for energy transduction. The best characterized RND transporter is AcrB, whose structure has been solved both in *apo* and *holo* forms, with bound substrates and inhibitors [23]. Structurally,

AcrB is an asymmetric homotrimer with each protomer comprising three domains: (i) a trans-membrane (TM) domain in the IM, where energy conversion takes place via proton coupling; (ii) a pore (porter) domain located in the periplasm, where recruitment and transport of substrates is believed to occur (although recent works pointed to additional entry gates at/or underneath the TM/pore interface [24,25]); and (iii) a periplasmic funnel domain, which connects the RND transporter to the OM channel protein via the assembly of the MFP in the complete pump (Fig. 2A). Apparently, substrate transport in these proteins follows a "functional rotation mechanism" in which concerted cycling of the protomers occurs through any of the so far identified asymmetric states: Access or Loose (L), Binding or Tight (T), and Extrusion or Open (O) [26,27]. A recent investigation demonstrated that the occurrence of such functional states might depend on the assembly of the tripartite machinery, whereby a chaperone-like complex between the OMF and the MFP allosterically controls the activity of the RND transporter [28].

Two drug-binding pockets, named proximal (or access, AP) and distal (or deep binding, DP), were previously identified in AcrB as the main sites contributing to transport of substrates [26,29]. These pockets are separated by a glycine-rich loop (or switch loop) whose flexibility is believed to be key for drug entry into the DP [30]. The latter appears to be visited during extrusion by all captured compounds (Fig. 2). The pocket comprises the so-called hydrophobic trap, HT, (lined by residues F136, F178, F610, F615, and F628), which is a critical recognition site for several inhibitors [31,32]. In addition to the periplasmic entry pathway leading from the AP to the DP, multiple entry channels have been proposed over the years (Fig. 2). These channels are thought to contribute to the polyspecificity of AcrB [24,25,33]. A prominent role of water molecules was put forward in stabilizing the binding of inhibitors [34] and in ensuring continuous substrate hydration on the inner surface of the channel leading from the DP to the funnel domain [35]. Thus, while unrelated drugs are sequestered by different entrance gates, screening specific interactions by water could enable smooth poly-specific transport within a unique duct.

RND efflux systems of Gram-negative bacteria have distinct but complementary substrate preferences leading to extrusion of most clinically relevant antibiotics from the bacterial cell (Fig. 3A). A prominent example is represented by the relevant differences in the substrate specificities of the clinically important RND transporters MexB, MexF and MexY of *P. aeruginosa* [36–40]. The substrate specificity of MexB is very similar to that of AcrB of *E. coli* as both proteins transport macrolides such as erythromycin, most beta-lactams such as carbenicillin, novobiocin, etc. In contrast, MexF is the most efficient in efflux of trimethoprim, chloramphenicol and fluoroquinolones, but not so much against other antibiotics. In addition to other antibiotics, MexY is the only pump which is also effective against aminoglycosides such as gentamicin or tobramycin (Fig. 3A).

These substrate preferences, however, are conditional and change depending on the permeability properties of the OM and the expression levels of these pumps. Clinical isolates overproducing either one of the MexAB-OprM, MexEF-OprN or MexXY-OprM gain resistance to a broad spectrum of antibiotics, although only the overexpression of MexXY confers clinical levels of resistance to aminoglycosides in the absence of other mechanisms [41]. At the same time, hyper-porination of the OM diminishes this polyspecificity due to

the overproduction of efflux pumps without affecting the transport of specific substrates [14,42]. This conditional polyspecificity implies the same underlying mechanism: a synergistic relationship with the OM barrier and structural flexibility of binding pockets and tunnels. On the other hand, the preferred substrates are pumped out with high efficiency, requiring only low efflux pumps expression levels and pointing to specific interactions and transport mechanisms [14]. Disentangling poly-specific and specific mechanisms in RND transporters is crucial for development of effective efflux pump inhibitors (EPIs).

Approaches to inhibition: stop the machine or prevent its assembly.

Several strategies to reduce active efflux have been proposed and pursued over the years and these can be grouped into three mechanistic classes: 1) prevention of efflux pump expression [43], 2) inhibition of efflux complex assembly [44,45], and 3) inhibition of fully assembled functional efflux pump [46–48] (Fig. 1B). The largest number of discovered EPIs are those acting on a fully assembled pump, specifically its RND component. Such EPIs are as structurally diverse as efflux substrates and can be identified using a variety of screening approaches. The first EPIs identified are cationic peptidomimetics able to penetrate the OM of Gram-negative bacteria and inhibit activities of various efflux pumps (Rempex compounds) [49] (Fig. 3b). Pyranopyridines were screened from large libraries and are potent inhibitors of AcrB and similar pumps in *Enterobacteriales* [31]. More than forty new efflux inhibitors, likely substrates of AcrB, were recently identified from two compound libraries selected for their high chemical and pharmacological diversity [48]. At least six structural classes of EPIs were singled out using computational methods with focused and pre-filtered compound libraries and *E. coli* AcrA as a target [44,50]. A series of 4-substituted 2-naphthamide derivatives [51] and compounds belonging to a series of piperazine arylideneimidazolones [52] were shown to potentiate the action of antibiotics by targeting the AcrAB-TolC in *E. coli*. Three chemical classes of inhibitors were identified using in-cell screening approaches [53].

Surprisingly, many of these EPIs are also good substrates of RND pumps [54,55], raising intriguing questions on how the two orthogonal properties co-exist within the same chemical scaffold and why EPIs do not inhibit their own efflux. EPIs that are also substrates typically act through a competitive inhibition mechanism, which is not particularly effective for specific interactions, and even less so for inhibition of poly-specific transporters. In a classical competitive inhibition, EPIs are expected to be effective at low concentrations of substrates and lose their potency at saturating concentrations. It is important to emphasize that biochemical analyses are very challenging with efflux pumps and much of the current understanding of the mechanisms of EPIs is based on measurements of inhibition of bacterial growth. Synergy with the OM and cell killing mechanisms are crucial in the interpretation of such findings but often are ignored.

The best characterized and the most effective EPIs-substrates interact with the DP of RND pumps and are thought to inactivate the pump through the binding inside the HT of the DP [32,34]. Peptidomimetic, pyridopyrimidine derivative ABI-PP and pyranopyridine EPIs (Fig. 3b), which share aromatic moieties able to form stacking bonds within the HT, apparently act by this mechanism. Importantly, the hydrophobicity or aromatic bonding alone are poor

predictors of the ability of compounds to act as EPIs-substrates. As an example, Rempex compounds are polar and positively charged molecules, the features characteristic for aminoglycoside antibiotics as well. Yet, Rempex compounds are both the substrates and inhibitors of MexB, while aminoglycosides are expelled only by MexY.

To explain inhibition of efflux, binding of EPIs-substrates in the DP was proposed to prevent conformational transitions in the transporter [32,34,56,57], the mechanism distinct from the classical competitive inhibition. Indeed, structural analyses showed that pyridopyrimidine derivative ABI-PP, as well as pyranopyridines such as MBX-2319, bind tightly to the HT [32,34]. More recently, cryo-EM analyses of the fully assembled AcrAB-TolC showed that a pre-treatment with a pyranopyridine inhibitor MBX-3132 changed the distribution of AcrB conformers with the majority of the apo-AcrB seen in the LLL state, whereas inhibitor-bound AcrB was found predominantly in the symmetric TTT conformation [58]. However, if the blockage of the conformational rotation in AcrB is indeed the mechanism of inhibition, how could EPIs also be substrates that are pumped out from the cell? It seems that the answer to this question is in the finding that many substrates also interact with the HT, but this binding does not inhibit the transporter. It is possible that when two competing substrates enter the DP, those with affinities to the HT are expelled better, whereas the delayed efflux of the second compound is manifested in the apparent inhibition of the substrates that interact weakly with the HT.

The two mechanisms of inhibition can be distinguished in microbiological and biochemical assays. Interference with the conformational transitions in AcrB is expected to affect all substrates of a transporter and such EPIs cannot be its own substrates. In contrast, for competitive inhibitors, the inhibitory effect will vary depending on a paired substrate, and the inhibitor will be recognized by the pump as a substrate. It is more challenging however, to identify physicochemical features in compounds that are distinctly associated with efflux substrates, inhibitors and avoiders.

Recently, these authors analyzed a set of 260 Rempex compounds that have potent antibacterial activity against *P. aeruginosa* [59]. Growth-dependent and -independent assays were used to establish propensities of compounds to inhibit efflux, to be pumped out or to avoid efflux pumps. Surprisingly, depending on chemical modifications, some of the Rempex compounds were found to be either exclusively substrates or EPIs, whereas others comprised both properties in the same scaffold or were efflux avoiders/non-EPIs. Machine learning models using physicochemical and efflux descriptors showed that compounds with properties of substrates and EPIs cannot be readily distinguished from each other based on these descriptors, because both interact with the same sub-sites on the MexB transporter. However, efflux avoiders can be distinguished from EPIs-substrates. The derived efflux avoidance and inhibition models indicated that avoiders and EPIs-substrates exhibit different binding affinity with the AP and DP of MexB (Fig. 4). Among the physicochemical properties distinguishing the two classes are molecular shape (represented by acylindricity, measuring deviation from cylindrical symmetry), amphiphilicity (represented by anisotropic polarizability) and partition coefficient logD (Fig. 4). The propensity to be an EPI-not-avoider increases with increasing acylindricity and anisotropic polarizability but decreases with increasing partition coefficient and lipophilicity. The above models were shown to be

predictive of such properties among compounds with unrelated chemical scaffolds [59], and the developed protocol can be used to effectively optimize efflux avoidance and inhibition.

Conclusions

The majority of RND transporters in Gram-negative bacteria are poly-specific, albeit to different degrees, suggesting that polyspecificity is an intrinsic property of their structures. Synergy with the low permeability barrier of the OM and variable entry tunnels leading to the DP are major underlying mechanisms of this polyspecificity. Their invariable presence in bacterial genomes supports the notion that the polyspecificity of RND transporters is a gift enabling environmental versatility and persistence of Gram-negative bacteria. On the other hand, identification of various EPIs from several structural classes suggests that such polyspecificity could also be a curse, because substrates are either already EPIs or can be converted into such through focused manipulations. Further characterization of specific and poly-specific mechanisms in RND transporter and identification of physicochemical properties distinguishing substrates and EPIs could potentially lead to new small molecule therapeutics controlling the activity of multidrug efflux pumps.

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List of abbreviations

RND	resistance nodulation and cell division
IM	inner membrane
OM	outer membrane
OMF	outer membrane factor
MFP	membrane fusion protein
TM	trans-membrane
AP	access pocket
DP	distal pocket
EPI	efflux pump inhibitor
HT	hydrophobic trap

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Highlights

- Polyspecificity of bacterial RND efflux pumps is the key to environmental versatility and antibiotic resistance of Gram-negative bacteria.
- Mechanisms to achieve polyspecific transport include synergy with the outer membrane, multiple entry channels, multifunctional binding sites and water-mediated substrate translocation.
- Inhibitors of RND transporters act by competing with substrates or by preventing conformational transitions.
- Machine learning models using physico-chemical and efflux descriptors represent a promising tool to identify specific and poly-specific features of ligand-RND interactions.

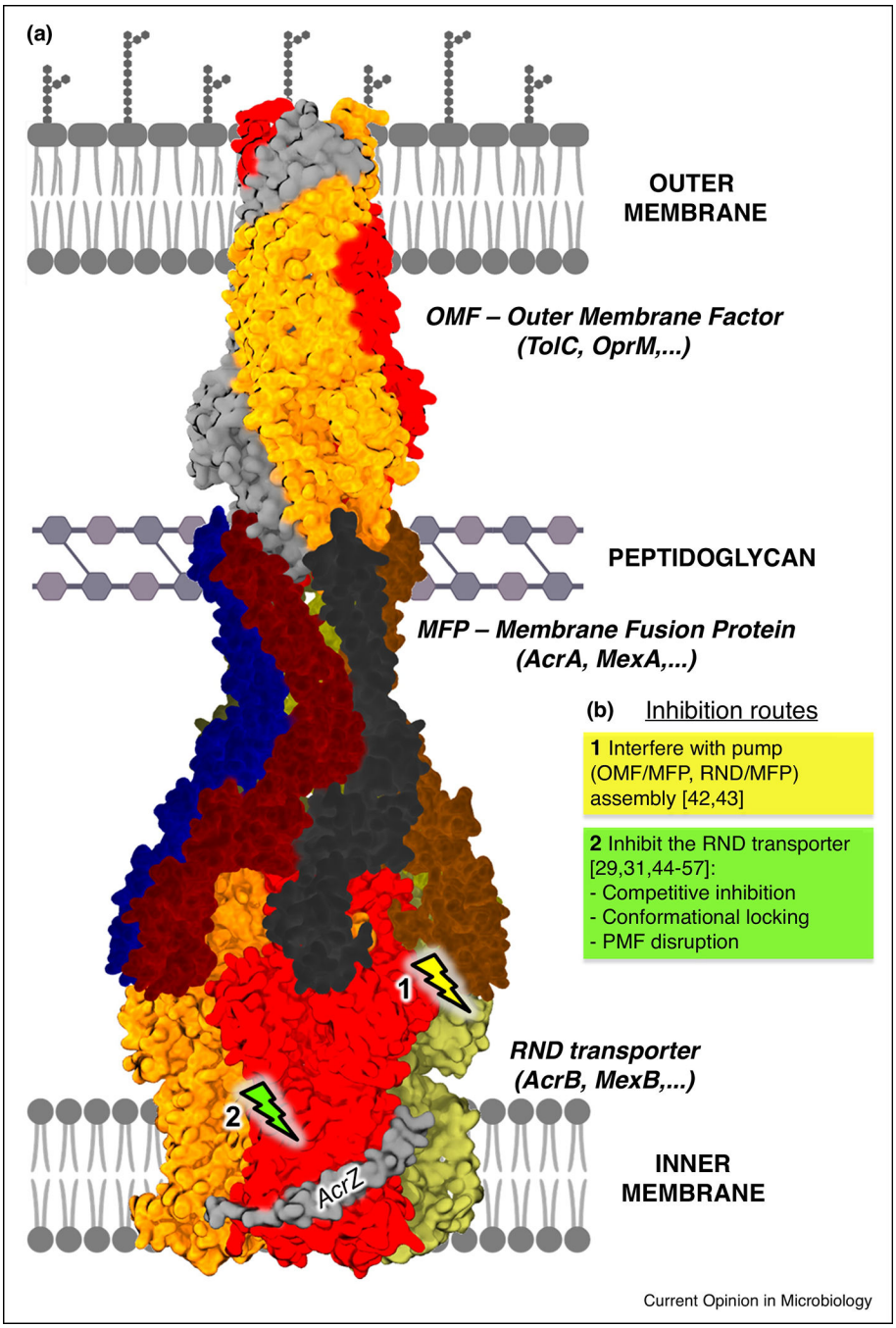


Figure 1.

A) Structure of the fully assembled AcrAB(Z)-TolC efflux pump of *E. coli* [PDB ID 5O66, [58]]. For each component, monomers are shown as molecular surfaces colored differently. Numbered lightnings indicate very approximately the targets for different inhibition strategies listed in **B**. Created with VMD1.9.3 and BioRender.com.

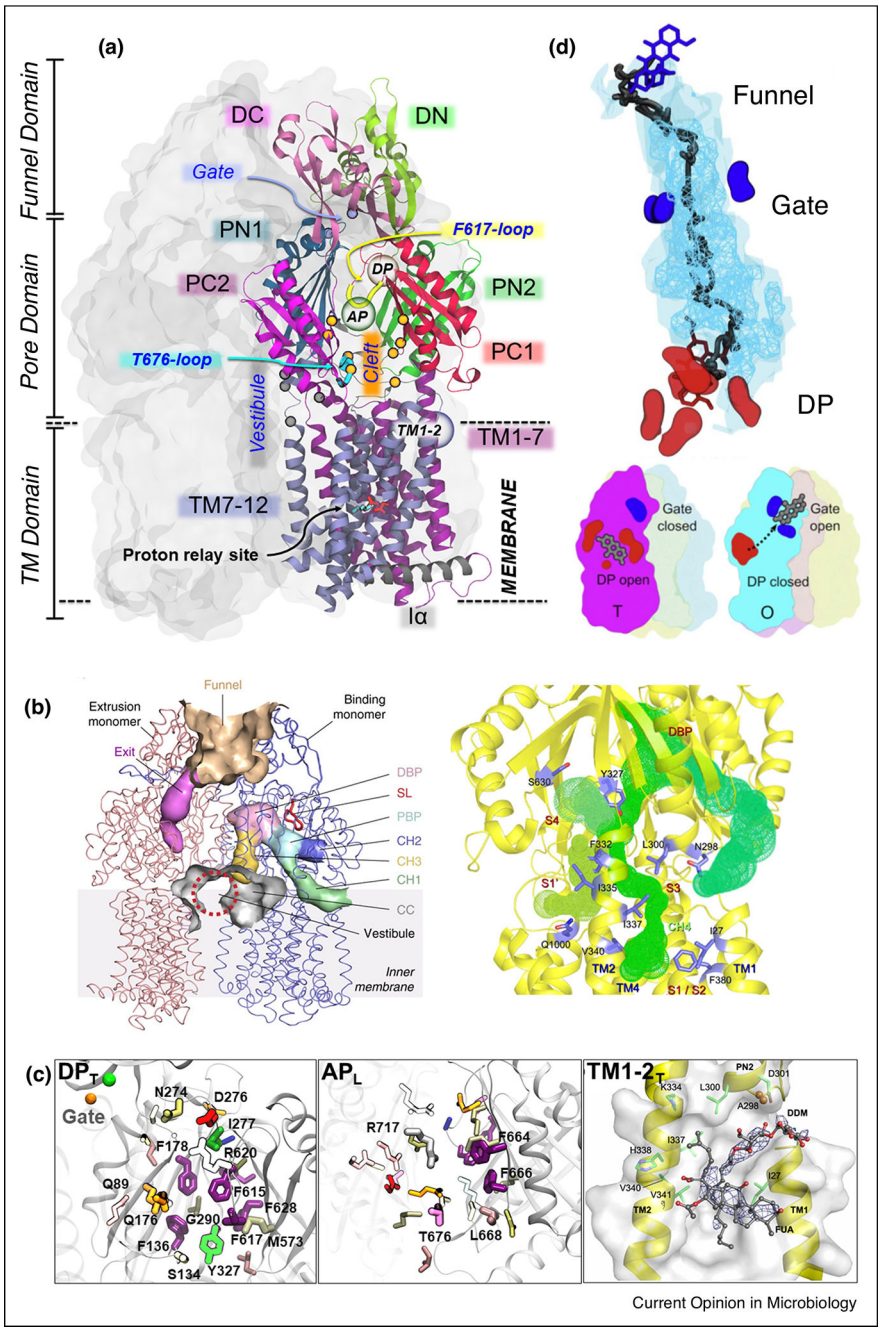


Figure 2.

A) Structure of the AcrB transporter of *E. coli* (PDB ID 4DX5,[29]) (modified from ([60]). Subdomains and secondary structural elements of the T protomer are shown in ribbons colored differently, and key elements putatively related to function are highlighted. The L and O monomers are shown as transparent surfaces. Transparent spheres indicate the approximate positions of TM1–2 (blue), access (green) and distal (red) binding pockets as deduced from co-crystallyzed structures. Residues D407, D408 and K940 lining the proton relay pathway within the TM region are shown as sticks colored according to their type (red and cyan for D and K residues respectively); **B)** Putative entry channels detected to date.

Left (adapted from [24]): Vestibule, CH1 to CH3, shown as solid channels colored differently within the T protomer. The exit Gate opening towards the Funnel Domain in the O protomer is also shown. Right (adapted from [25]): CH4 entry channel, with sidechains of lining residues shown as violet sticks; **C)** Residues lining the DP_T, AP_L (adapted from [61]) and TM1–2_T sites (adapted from [25]) are shown as sticks. The subscripts T and L indicate the Tight and Loose protomers, respectively. Phenylalanines lining the HT within the DP are colored in violet. **D)** Spatial distribution function isosurfaces (isovalues of 5 and 1 with respect to the average value in bulk water are shown as light cyan nets and transparent surfaces, respectively) of water oxygen atoms within the transport channel leading from the DP to the Funnel domain via the exit Gate, as seen in molecular dynamics simulations of the transport of doxorubicin (red and blue sticks at the beginning and end of the simulation, respectively) during the LTO → TOL conformational change (shown schematically below). The pathways traced by the center of mass of the drug are displayed as dark gray tubes. Residues lining the HT and exit Gate are shown as red and blue surfaces, respectively (adapted from [35]).

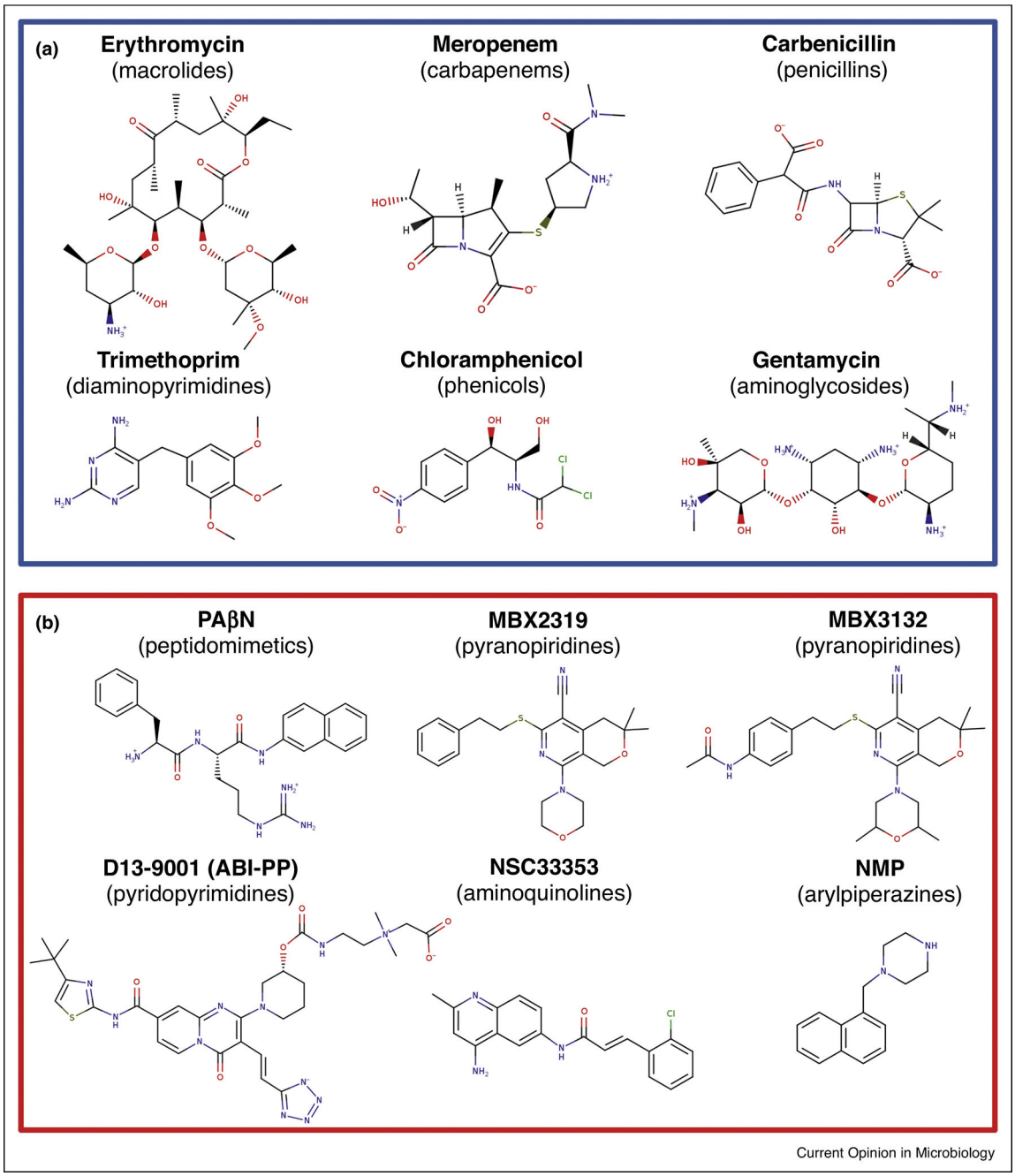
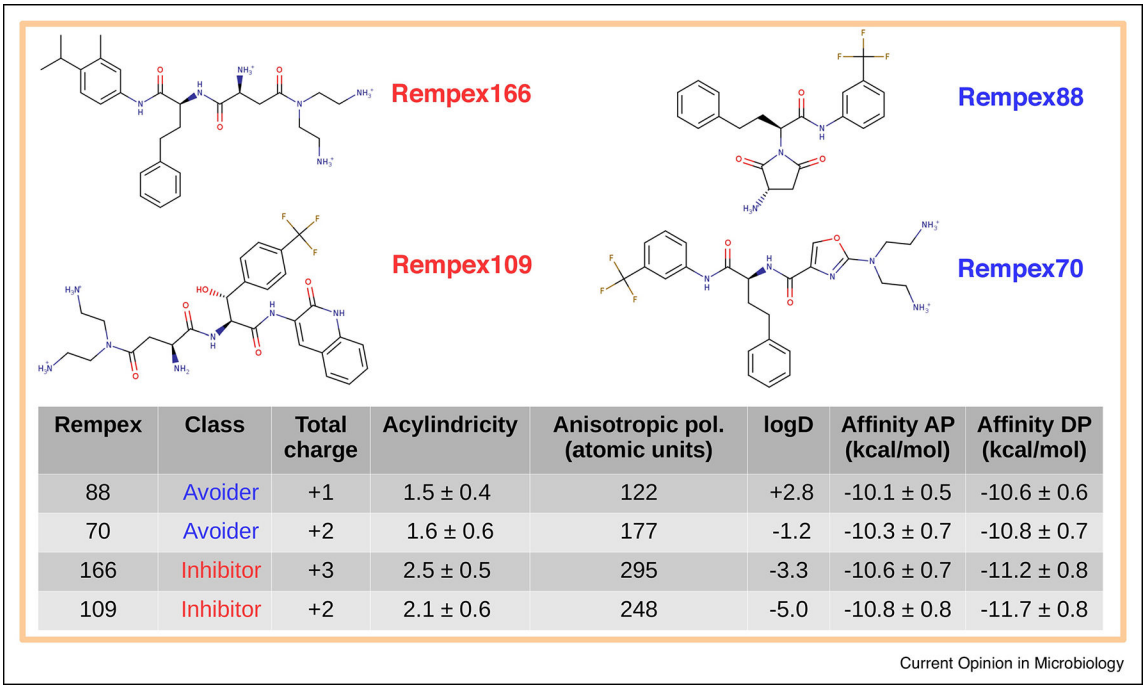


Figure 3.

Representative antibiotics (**A**) and EPIs structures (**B**).



Rempex	Class	Total charge	Acylindricity	Anisotropic pol. (atomic units)	logD	Affinity AP (kcal/mol)	Affinity DP (kcal/mol)
88	Avoider	+1	1.5 ± 0.4	122	+2.8	-10.1 ± 0.5	-10.6 ± 0.6
70	Avoider	+2	1.6 ± 0.6	177	-1.2	-10.3 ± 0.7	-10.8 ± 0.7
166	Inhibitor	+3	2.5 ± 0.5	295	-3.3	-10.6 ± 0.7	-11.2 ± 0.8
109	Inhibitor	+2	2.1 ± 0.6	248	-5.0	-10.8 ± 0.8	-11.7 ± 0.8

Figure 4.

Structures of representative Rempex compounds with associated physicochemical descriptors, including average binding affinities for the AP and DP of MexB as predicted by molecular docking [59].