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Efflux systems in bacterial pathogens: An opportunity for therapeutic intervention? An industry view

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ABSTRACT

The efflux systems of bacteria protect cells from antibiotics and biocides by actively transporting compounds out of the cytoplasm and/or periplasm and thereby limit their steady-state accumulation at their site(s) of action. The impact of efflux systems on the efficacy of antibiotics used in human medicine and animal husbandry is becoming increasingly apparent from the characterization of drug-resistant strains with altered drug efflux properties. In most instances, efflux-mediated antibiotic resistance arises from mutational events that result in their elevated expression and, in the case of efflux pumps with broad substrate specificity, can confer multi-drug resistance (MDR) to structurally unrelated antibiotics. Knowledge of the role of efflux systems in conferring antibiotic resistance has now been successfully exploited in the pharmaceutical industry and contributed, in part, to the development of new members of the macrolide and tetracycline classes of antibiotics that circumvent the efflux-based resistance mechanisms that have limited the clinical utility of their progenitors. The therapeutic utility of compounds that inhibit bacterial drug efflux pumps and therein potentiate the activity of a co-administered antibiotic agent remains to be validated in the clinical setting, but the approach holds promise for the future in improving the efficacy and/or extending the clinical utility of existing antibiotics. This review discusses the potential of further exploiting the knowledge of efflux-mediated antibiotic resistance in bacteria toward the discovery and development of new chemotherapeutic agents.

1. Introduction

Systematic analysis of bacterial genomes substantiates the importance of membrane transport systems in prokaryotic evolution and physiology [1–5]. Genes classified as encoding putative drug efflux systems, based on their homology to functionally characterized antibiotic transporters, have been estimated to comprise 3–24% of the full transporter complement [4,6]. Fig. 1 shows a diagrammatic representation of the five different families of antibiotic efflux systems identified to date that encompass two mechanistically distinct types: primary transporters that couple drug extrusion from the cell with ATP hydrolysis and secondary transporters energized by trans-membrane electrochemical gradients of either protons or sodium ions.

Antibiotic efflux systems that couple drug export with ATP hydrolysis are all members of the ATP-binding cassette (ABC) superfamily of transporters distributed throughout both prokaryotic and eukaryotic genera [7]. ABC transporters are typically comprised two hydrophobic membrane localized domains and two hydrophilic domains localized at the cytoplasmic interface and that bear signature motifs

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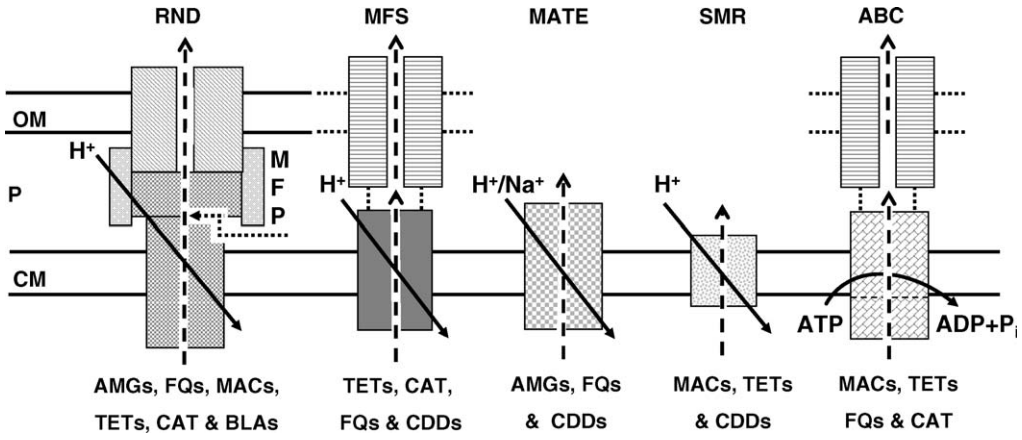

Fig. 1 – Bacterial antibiotic drug transporters. The figure shows diagrammatic representations of the five structural classes of antibiotic drug transporters and for each lists the antimicrobial substrate classes identified to date. Abbreviations employed—MFS: major facilitator superfamily; RND: resistance-nodulation-division; MATE: multidrug and toxic compound extrusion; SMR: small multi-drug resistance; ABC: ATP-binding cassette. OM: outer membrane; P: periplasm; CM: cytoplasmic membrane; MFP: membrane fusion protein; TETs: tetracyclines; CAT: chloramphenicol; FQs: fluoroquinolones; CDDs: cationic dyes and detergents; AMGs: aminoglycosides; MACs: macrolides; BLAs: β-lactams. See text and recent reviews [9,98–100] for more specific details.

involved in ATP binding and hydrolysis. The individual domains can be expressed as separate proteins or expressed as multi-domain polypeptides in various configurations [2]. In bacteria, transporters of the ABC class typically possess high specificity for substrates that include sugars, amino acids, metallic cations, organo-iron complexes, vitamins as well as antibiotics. In humans, ABC proteins that confer resistance to antitumor agents include P-glycoprotein (MDR1) and the multi-drug resistance proteins 1 and 2 (MRP1 and MRP2).

Antibiotic efflux systems classified as secondary transporters include members of the major facilitator superfamily (MFS), the resistance-nodulation-division (RND) superfamily, the small multi-drug resistance (SMR) family of the drug metabolite transporter (DMT) superfamily, and the multidrug and toxic compound extrusion (MATE) family of the multi-drug/oligosaccharidyl-lipid/polysaccharide flippase (MOP) superfamily. Of these, the RND, SMR and MATE classes are unique to prokaryotes whereas MFS transporters are broadly distributed in both prokaryotes and eukaryotes and, as with the ABC class, are engaged in the import and export of a variety of diverse substrates.

The distribution of the various classes of antibiotic efflux systems between different bacteria in part reflects their cell surface architecture. Gram-negative bacteria and mycobacteria have both inner membrane (IM) and outer membrane (OM) with an intervening periplasmic space. Export of antibiotics to the extracellular space therefore requires transport through both membranes and is carried out by dedicated multi-component systems wherein the inner membrane pump protein is transiently or permanently associated with an outer membrane channel protein [8–10]. For a number of the best characterized RND class efflux pumps, tri-partite transport systems involve specific interactions between the IM pump protein, an OM channel protein and a so-called membrane fusion protein (MFP). Well-characterized examples of such include the AcrA–AcrB–TolC system of *Escherichia coli* and the MexA–MexB–OprM system of *Pseudomonas aeruginosa*. The organization of genes encoding the three separate components of some tri-partite RND systems into operons [3,10] suggests they have specific cellular roles and that the associations between the IM and OM components are highly specific. In the case of MFS and ABC class efflux pumps of Gram-negatives and mycobacteria, coupling between the IM pump protein and an OM porin channel also appears to involve associations with a MFP but only a few examples of discrete tri-partite systems, like the macrolide-specific MacA–MacB–TolC ABC class transporter of *E. coli* [11], have been reported to date. In other cases, it is apparent that OM channel proteins (or porins) are more promiscuous in their associations with IM efflux pumps.

In antibiotic-producing bacteria, it is clear that efflux systems confer self-immunity to the antibiotic(s) produced [12–14] and in other cases serve to protect bacteria from antibiotics produced by their natural environmental competitors [5]. However, the majority of bacterial efflux systems appear to have roles in other processes like intercellular communication [15–18] or the export of toxic metabolic by-products [17,19–21] and are likely co-opted in conferring intrinsic or acquired resistance to antibiotics in pathogens in the clinical setting. Indeed, the apparent essentiality of some putative drug efflux systems for growth in vitro [22] and/or normal virulence in vivo [17,23,24] in the absence of antibiotic challenge must reflect unknown critical roles in metabolism, physiology and/or virulence.

Recent studies employing functional genomics methods [25,26] or microarray analysis [27–31] highlight the intrinsic role of efflux systems in determining the basal or inducible level of sensitivity of bacteria to antibacterial agents. However, characterization of the genetic basis underlying antibiotic resistance in clinical isolates, combined with experimental

genetics studies, highlight the numerous pathways by which mutational events can give rise to strains with altered efflux properties. These include (i) the constitutive elevation of the expression of efflux genes through direct and indirect mechanisms, (ii) the expression of efflux pumps with apparent altered substrate affinity or specificity, and (iii) the acquisition of mobile genetic elements that express non-native efflux pumps.

Recognition of the central role that efflux systems play in limiting the clinical utility of antibiotics [6,32,33], combined with the apparent dearth of new structural classes of antibiotics in development at a time when MDR forms of key pathogens are becoming increasingly prevalent [34,35], has triggered interest in exploiting knowledge of antibiotic efflux toward the discovery and development of new chemotherapeutic agents.

2. Impact of efflux technologies on antibiotic discovery and development

Growth inhibition assays have traditionally been used to identify antimicrobial substances from natural product sources or synthetic compound collections and have yielded all of the antibiotic agents currently approved for human use. In light of the increased antibiotic sensitivity of efflux-defective mutants, there has been considerable interest in deploying efflux-defective mutants in high-throughput screens (HTS) to identify antimicrobial substances that would be missed in conventional screens employing wild-type strains [36,37]. Similarly, the availability of bacterial efflux pump inhibitors (EPI's – see Section 3) provides additional new tools for use in cell-based screens through effectively re-sensitizing the target bacteria to test compounds [6,33,37]. While the further development of compounds identified by these approaches requires that chemistry efforts be directed towards the circumvention of the specific efflux system(s) that limit activity against wild-type strains, they clearly hold promise for the discovery of novel antibacterial leads.

The clinical utility of the tetracycline [38], macrolide [39,40] and fluoroquinolone [32,41,42] classes of antibiotics has been significantly impacted by the emergence and dissemination of efflux-based resistance traits. However, characterization of the specific efflux systems involved in conferring clinical resistance has provided a knowledge base to guide efforts towards the generation of novel derivatives that circumvent them. In the tetracycline and macrolide classes, semi-synthetic chemistry efforts have yielded compounds of the so-called glycylcycline [38,43–45] and ketolide [40,46–48] classes, respectively, that differ from their progenitors in no longer being high affinity substrates for pertinent efflux pumps and therein have superior activity against clinical strains bearing activated efflux systems. Specifically, tigecycline circumvents a series of MFS class tetracycline-specific efflux proteins of both Gram-negative and Gram-positive pathogens [43,45,49] while telithromycin has significantly improved activity versus clinical isolates with elevated macrolide efflux via the MefA/E [50] and AcrAB systems [48]. Finally, in the case of the fluoroquinolones, the overall hydrophilicity [51,52] and bulk [53–55] of compounds affects their sensitivity to specific efflux pumps and the apparent SAR's delineated have contributed in the identification of novel development candidates that are less prone to efflux [56].

Based on the successes apparent to date, it seems likely that the use of engineered strains with altered efflux properties will continue to be employed in both drug discovery and development efforts. For instance, the clinical utility of the oxazolidinone class of antibiotics is essentially restricted to Gram-positive pathogens as the activity of the compounds against key Gram-negative pathogens is compromised at the level of intrinsic efflux [57]. However, directed efforts could be made towards the identification of oxazolidinones that circumvent the relevant Gram-negative efflux system(s) and therein attain an improved spectrum of activity.

3. Efflux pump inhibitors as antibiotic potentiating agents

There has also been significant interest in the development of a new class of antibiotic-potentiating agents that act as specific efflux pump inhibitors [6,33,58]. Such agents would be co-formulated (or co-administered) with the appropriate antibiotic(s) in an adjunctive therapy approach akin to the current combinations of β-lactam antibiotics with cognate β-lactamase inhibitors. Further, as efflux-based resistance often represents one of the 'first-step' events in mutational pathways toward high-level antibiotic resistance, it is anticipated that EPI's may have added clinical benefit in suppressing the rate of resistance development [6,33]. Efforts toward the identification of EPI's can be divided into two broad categories based on the discovery approach employed.

First, as antibiotics are specific substrates for their cognate efflux systems, a number of chemistry-based programs have focused on the derivatization of antibiotic scaffolds to yield EPI's that may not retain appreciable antimicrobial activity but which can be combined in a synergistic fashion with another member of the same antibiotic class. In this category, the best characterized examples are a series of tetracycline derivatives substituted in position 13, including 13-cyclopentylthio-5-hydroxy tetracycline (13-CPTC) [59–61]. However, despite early promise, no clinical development candidates as EPI's of this category are apparent from the published literature.

A second approach entails cell-based screens in which one (or more) specific antibiotics is included in the growth media at a concentration sub-inhibitory for bacterial growth, using either wild-type or strains with elevated efflux, to identify potential EPI's in a de novo fashion that are capable of re-sensitizing the test strain to the antibiotic [36,37]. Examples of EPI's identified in this fashion from screens of libraries comprised natural product extracts or synthetic compounds include (i) alkylaminoquinolines that inhibit the AcrAB pump of Gram-negative bacteria and increase sensitivity to a series of structurally unrelated antibiotics [62,63], (ii) arylpiperazines that inhibit the AcrAB and AcrEF pumps of *E. coli* and potentiate the activity of fluoroquinolones [64], (iii) a series of flavonolignans and flavones that inhibit the NorA MDR pump of *Staphylococcus aureus* [65–67], (iv) a series of small molecule inhibitors of the *S. aureus* NorA MDR pump that

possess chemical substructures inherent to the core nucleus of reserpine including compounds INF-55 (an indole) and INF-271 (a bi-phenyl urea) and INF-392 [68], (v) polyacylated neohesperidosides that were found to potentiate fluoroquinolones and other antimicrobials in *S. aureus* [69], and (vi) specific inhibitors of tetracycline efflux including Ro-07-3149 [70,71], UK-57-562 [58] and Nocardamine [71]. However, despite the number and variety of these discovery programs, the molecular mode of action of any of these compounds as specific pump inhibitors has yet to be established in detail and the results of chemistry efforts directed toward their systematic optimization as EPI's have not been reported.

The most extensively investigated series of EPI compounds resulted from a collaborative effort between Microcide Pharmaceuticals and Daiichi Pharmaceuticals. The progenitors of this series were discovered in an HTS as potentiators of fluoroquinolone activity against *P. aeruginosa* and subsequently characterized as inhibitors of the MexAB, MexCD, MexEF and MexXY RND-class pumps [72,73]. One early lead compound, a low molecular weight dipeptide amide (L-Phe-L-Arg-β-napthylamine) designated MC-207,110, showed minimal intrinsic antibacterial activity but potentiated the in vitro activity of levofloxacin by eight-fold at 10 μg/ml [72,73]. Subsequent chemistry efforts focused on both further establishing structure–activity relationships pertaining to efflux pump inhibition while addressing problems pertaining to biological instability [74,75], low aqueous solubility and high serum protein binding [76,77] and a toxicity observed in rodents [78]. These combined efforts have resulted in a second generation of leads with greatly improved physical properties and activity in the potentiation of fluoroquinolones against *P. aeruginosa* in rodent infection models [76,77].

A further group of EPI's that have been evaluated as potential start-points for the development of antibiotic-potentiating compounds have their origin in other therapeutic areas. Examples include (i) the plant alkaloid, reserpine, an antihypertensive and neuroleptic agent that was originally identified as an inhibitor of the *Bacillus subtilis* Bmr [79,80] and *S. aureus* NorA [81] MDR pumps and has since been broadly employed in studies of antibiotic efflux, (ii) calcium channel blockers like verapamil [82] and H^+/K^+ ATPase pump inhibitors like omeprazole and lansoprazole [83] that likely perturb bacterial MDR efflux systems by affecting the trans-membrane proton gradient, (iii) a series of phenothiazine and thioxanthene compounds, that are dopamine receptor antagonists and calmodulin inhibitors employed as neuroleptic and antiemetic agents, that were found to inhibit one or more MDR efflux systems in *S. aureus* [84], (iv) P-glycoprotein inhibitors including GG918 [85], and (v) Biricodar (VX-710) and Timcodar (VX-853)—both small molecule inhibitors of the human P-glycoprotein and MRP-1 MDR transporters [86]. In the latter case, it is suggested that the minimal toxicities of Biricodar that were observed in humans when evaluated as an MDR antagonist indicates that cross-reactivity between antibiotic EPI's and related human transporters may not manifest toxicities in the clinic [86]. However, it should be noted that most of these EPI compounds only show appreciable antibiotic-potentiating activity at concentrations significantly exceeding those used clinically in their other therapeutic contexts.

Finally, it has been proposed that down-regulation of the expression of genes encoding antibiotic efflux pumps may provide an alternate route towards the development of antibiotic-potentiating agents active via modulation of the efflux properties of target bacteria. This possibility stems largely from studies of ligand-gated transcription factors wherein substrates for specific efflux pumps also serve as ligands for transcriptional regulators of the expression of the cognate efflux pump gene(s) [6,33,58,87]. To date, no success in the identification of efflux modulators of this type is apparent from the published literature; however, the availability of high-resolution structural information for regulators of clinically relevant efflux systems [87,88] provides new potential opportunities in the design and/or optimization of such agents.

In the treatment of bacterial diseases, one drug development program involving co-administration of an EPI with an antibiotic agent has reached human clinical trials. In this case, an aerosolized formulation of the EPI compound MP-601,205 is being combined with ciprofloxacin for the treatment of pulmonary exacerbations in cystic fibrosis patients in a phase II trial being conducted by Mpex Pharmaceuticals with funding from the Cystic Fibrosis Foundation. Neither the structure nor a specific mode of action of the MP-601,205 compound has been disclosed. However, published data from in vitro and in vivo studies of other EPI's suggests that effective inhibition of the endogenous (or mutationally elevated) activity of fluoroquinolone efflux pumps of the RND class can result in hypersensitization of *P. aeruginosa* to ciprofloxacin to a level consistent with improved efficacy [6,33,58,72–75,78] and may also impact de novo resistance development [73,89].

4. Conclusions and future prospects

The emergence of MDR and pan-resistant variants of some key nosocomial pathogens, coupled with an apparent weak pipeline of novel antibiotic agents in development, dictates that no feasible routes towards extending the efficacy and/or future utility of existing agents be ignored. However, while knowledge of drug efflux pathways has been productively applied toward the discovery and/or development of new antibiotic agents, there is little substantive data that establishes EPI's as a class of antibiotic-potentiating agents that have a clear path towards clinical utility. In considering the future prospects for EPI's, a number of apparent pitfalls in the approach are apparent.

First, some broad-spectrum antibiotics are subject to efflux by multiple different classes of structurally unrelated pumps. In the case of the fluoroquinolones, efflux pumps of the ABC, RND, MFS and MATE families have all been reported to contribute to innate or mutationally activated efflux. Hence, an EPI that would function as a true broad-spectrum potentiator of a fluoroquinolone would itself have to possess specificity consistent with effective inhibition of efflux pumps from multiple different families. For non substrate-competitive classes of EPI's, then this appears a priori to be a chemically intractable approach. For substrate-competitive EPI's, success in achieving true broad-spectrum coverage would likely require that the antibiotic (and EPI) is recognized

by different classes of efflux pump in a similar fashion. This limitation of the approach is exemplified in the case of tigecycline; while this recently approved agent circumvents the MFS-class of tetracycline-specific efflux systems that have limited the clinical utility of its clinical progenitors [43,49], it remains sensitive to both MATE [90] and RND [91–94] class efflux pumps.

Second, genetic studies indicate that the expression of efflux systems with overlapping substrate specificity may be regulated in a hierarchical fashion such that the impact of any one efflux pump on the overall efflux of a particular antibiotic may only be apparent under (i) environmental conditions wherein the hierarchical regulatory system is perturbed or (ii) mutational events disrupt the normal hierarchical regulatory system. For instance, genetic studies of fluoroquinolone, β-lactam and triclosan efflux systems in *P. aeruginosa* have revealed multiple redundant systems that appear to be regulated in a hierarchical fashion [95–97]. Hence, pharmacological modulation of any one efflux system with an EPI may not be sufficient as to change the overall efflux propensity of some antibiotics by certain bacteria to a critical level consistent with their pursuit as clinical therapeutic agents.

Third, it should be anticipated that target bacteria will respond to clinical challenge by EPI's (when used in combination with their cognate antibiotics) through the development of resistance mutations that diminish the efficacy of the EPI, as is apparent from laboratory studies [80]. Assuming such mutations do not affect efflux of the cognate antibiotic, then mutational restoration of the antibiotic efflux pathway will confer a selective advantage on the bacterial host strain. Hence, diagnostic tests would be needed wherein clinical microbiology laboratories can distinguish between resistance traits that affect the target-directed effect of an antibiotic and those that affect the activity of the cognate EPI.

Overall, EPI's hold promise for the development of a new class of antibiotic-potentiating agent that may extend the clinical utility of both existing and future antibiotics. However, the available data support the contention that the development of EPI's for use in combination with antibiotics targeted towards narrow spectrum clinical uses appears to represent the most likely achievable short-term goal.

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