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Practical applications and feasibility of efflux pump inhibitors in the clinic—A vision for applied use

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ABSTRACT

The world of antibiotic drug discovery and development is driven by the necessity to overcome antibiotic resistance in common Gram-positive and Gram-negative pathogens. However, the lack of Gram-negative activity among both recently approved antibiotics and compounds in the developmental pipeline is a general trend despite the fact that the plethora of covered drug targets are well-conserved across the bacterial kingdom. Such intrinsic resistance in Gram-negative bacteria is largely attributed to the activity of multidrug resistance (MDR) efflux pumps. Moreover, these pumps also play a significant role in acquired clinical resistance. Together, these considerations make efflux pumps attractive targets for inhibition in that the resultant efflux pump inhibitor (EPI)/antibiotic combination drug should exhibit increased potency, enhanced spectrum of activity and reduced propensity for acquired resistance. To date, at least one class of broad-spectrum EPI has been extensively characterized. While these efforts indicated a significant potential for developing small molecule inhibitors against efflux pumps, they did not result in a clinically useful compound. Stemming from the continued clinical pressure for novel approaches to combat drug resistant bacterial infections, second-generation programs have been initiated and show early promise to significantly improve the clinical usefulness of currently available and future antibiotics against otherwise recalcitrant Gram-negative infections. It is also apparent that some changes in regulatory decision-making regarding resistance would be very helpful in order to facilitate approval of agents aiming to reverse resistance and prevent its further development.

1. Efflux pumps and antibiotic resistance

The world of antibiotic drug discovery and development can be divided into three categories based on development stage, each riddled with and driven by the necessity to overcome antibiotic resistance in common Gram-positive and Gram-negative pathogens. Drug efflux is a common theme throughout, and a major determinant for the efficacy of antibiotics both old and new. Approaches to overcoming drug efflux offer new opportunities to combat antibiotic resistance development across the spectrum of drugs in clinical use.

The first category are antibiotics approved by the FDA from 1998 to 2005, including rifapentine, quinupristin/dalfopristin, moxifloxacin, gatifloxacin, linezolid, ceftidoren, ertapenem, gemifloxacin, daptomycin, telithromycin and tigecycline [1,2]. The second category are antibiotics currently in clinical trials. These are doripenem, ceftobiprole, dalbavancin, telavancin, ramaplanin, sitafloxacin and garenoxacin, all in Phase III trials, as well as more Phases I and II fluoroquinolones and β-lactams, the macrolide EP-420, the oxazolidinone ranbezolid, the dihydrofolate reductase inhibitor iclaprim, the peptide deformylase inhibitor LMB-415

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and the tetracycline analog PTK 0796 [3]. Finally, the third category consists of compounds at various stages of preclinical development, including more β-lactams, fluoroquinolones, oxazolidinones and ketolides [4] as well as new analogs in the less prevalent rifamycin [5] and lincosamide [6] classes. This category also contains several compounds with novel modes of action, such as the dual GyrB/ParE inhibitor VX-692 [7] and the FabI inhibitor API-1401 [8]. Until recent termination or suspension [9,10], this category included many more compounds with novel modes of actions—compounds targeting DNA-polymerase, DNA-ligase, t-RNA synthetases, enzymes essential in cell division as well as various essential metabolic and unexploited cell-wall synthesis enzymes [11,12].

Compounds from all three categories have one thing in common. Almost all have poor activity against *Pseudomonas aeruginosa* and other recalcitrant Gram-negative bacteria (such as *Acinetobacter* spp., *Stenotrophomonas maltophilia* and *Burkholderia cepacia*). In fact, most of them lack appropriate activity against any Gram-negative bacteria at all [13]. Only tigecycline, the most recently approved antibiotic [14,15], has potent in vitro activity against *Acinetobacter* spp. and *S. maltophilia*, but not *P. aeruginosa* or Proteae, and only the carbapenem doripenem, is active against *P. aeruginosa* [16,17], but not the strains producing metallo-β-lactamases. In this respect it is not different from the currently approved antipseudomonal carbapenems, imipenem and meropenem. This general trend in the lack of Gram-negative activity among both recently approved antibiotics and compounds in the developmental pipeline is rather remarkable considering the plethora of drug targets covered, both old and new, and the fact that most of the compounds have the potential to be truly broad-spectrum since they inhibit the activity of ''genomically correct'' targets that are well-conserved in both Gram-positive and Gram-negative bacteria.

It is now widely recognized that constitutive expression of efflux pumps encoded by house-keeping genes widespread in bacterial genomes is largely responsible for the phenomenon of intrinsic antibiotic resistance [18]. Antibiotic efflux was first discovered in 1980, when it was recognized as a mechanism for tetracycline resistance in enterobacteria [19]. Since then, it has been shown that almost all antibiotics are subject to resistance by efflux [20–22], and that antibiotic efflux can be mediated by more than one pump in a single organism [23–25]. Some bacterial efflux pumps selectively extrude specific antibiotics, while others, the so-called MDR pumps, expel from cells a variety of structurally diverse compounds [26]. In the latter case, a single bacterial protein may confer intrinsic resistance to multiple, structurally diverse antibiotics with different modes of action. In fact, most of the so-called ''Gram-positive'' antibiotics, many cited above, gain potent Gram-negative antibacterial activity when measured against mutant strains of *Escherichia coli* or *P. aeruginosa* lacking the major, constitutively expressed efflux pumps AcrAB–TolC or MexAB–OprM, respectively [27,28]. By the same token, bacteria can become clinically resistant to multiple antibiotics simultaneously due to overexpression of a single operon-encoding MDR pump, without acquisition of multiple specific resistance determinants or multiple target-based mutations. It is believed that dual targeting is an efficient way to dramatically reduce the rate of emergence of antibiotic resistance [10]. However, dual targeting will not solve the problem if resistance is mediated by overexpression of efflux pumps which protect both targets. Also it is noteworthy that, in general, the frequency of efflux-mediated resistance is higher than the frequency of resistance based on target alterations [29]. This is easily understood considering the fact that many efflux genes are under the control of negative regulators and their overexpression occurs as a result of loss of function mutations in corresponding repressor genes. The frequency of loss of function mutations is usually much higher than the frequency of change of function mutations. Loss of function will occur due to many nonsense and frame-shift mutations while only rare mutations in specific regions of an essential target gene product will maintain protein function with a decreased affinity to an inhibitor compound.

Multidrug resistance efflux pumps are expressions in both Gram-positive and Gram-negative bacteria. However, it is in Gram-negative bacteria where they exert their strongest effect in intrinsic and acquired antibiotic resistance, reaching as much as several orders of magnitude change in bacterial susceptibility. This is attributed to the combined effect of so-called trans-envelope efflux (whereby a toxic compound is captured in the periplasm and extruded directly into the external medium; see below) and reduced uptake across the Gram-negative outer membrane [21,30,31], which provides an effective barrier for both hydrophilic and hydrophobic compounds [32]. It comes as no surprise that efflux-mediated intrinsic and acquired resistance is well-documented even for the limited number of antibiotics (fluoroquinolones, β-lactams and aminoglycosides), which are available for the treatment of *P. aeruginosa* and similar recalcitrant Gram-negative bacteria [18,33].

These considerations provide a strong argument and rationale for preserving and significantly improving the usefulness of antibacterial agents by interfering with efflux pumps through small molecule inhibitors. Such inhibitors used in combination with antibiotics will increase antibacterial potency, expand the spectrum of antibacterial activity, reverse resistance and dramatically reduce the rates of resistance development. An alternative and potentially more elegant approach is to identify and develop compounds which avoid efflux pumps altogether. Indeed, there are several examples where this approach has been perfectly successful for Gram-positive bacteria. In the first example, the newer fluoroquinolones, levofloxacin, moxifloxacin, gemifloxacin, gatifloxacin and garenoxacin are not affected by the multidrug resistance pumps, NorA and PmrA, in *Staphylococcus aureus* or *Streptococcus pneumonia*, respectively [34–39]. It is believed they are sufficiently hydrophobic so that their rapid passive uptake overwhelms active efflux from the cell. Another possibility is that structural modifications resulting in increased hydrophobicity might directly contribute to the altered affinity for the transporter itself. Of note is that the affinity might be either lowered or enhanced. In the former case, the pump protein will not recognize the substrate and in the latter case the substrate will not efficiently dissociate from its binding site. The result, however, will be same: a decreased effect of efflux on antibiotic susceptibility. Indeed, sparfloxacin has been shown to non-competitively inhibit the

NorA transporter, which may be the reason why susceptibility to sparfloxacin is not affected by the NorA pump [40]. In the case of tetracycline derivatives tigecycline [41,42] and PTK 0796 [43] the mechanism of resistance to efflux is due to the lack of recognition by the tetracycline-specific transporters (TetA-D and TetK, M). Note, however, that the ''out-foxed'' efflux pumps mentioned above are either antibiotic-specific, or are MDR transporters from Gram-positive bacteria. The same antibiotics are still effectively extruded by MDR transporters from Gram-negative bacteria. In fact, almost all antibiotics studied in this regard are substrates of the Gram-negative transporters. The only notable exceptions are some representatives of the β-lactams [44–50]. The major feature which differentiates them from all other antibiotics is the periplasmic rather than cytoplasmic location of their targets, penicillin-binding proteins. Consequently, in order to kill bacterial cells they do not need to cross the inner bacterial membrane. It is hypothesized that this is the reason why some are not affected by efflux transporters [51,52]. By the same token, it is highly improbable to identify a compound that inhibits an intracytoplasmic target while not being recognized by one or another of the MDR transporters in Gram-negative bacteria. Unfortunately, the idea of making compounds more lipophilic to facilitate their uptake across the inner membrane will also not work in Gram-negative bacteria, since uptake of the very same compounds will be strongly reduced by the outer membrane [32,53]. Therefore, back to approach one, inhibition of the pumps by a separate chemical entity. First though, we need to get to know the enemy.

2. Efflux pumps as discovery targets

Five families of bacterial drug efflux pumps have been identified to date [54]. However, it is mostly members of a single resistance/nodulation/division super family (RND) that are implicated in clinically relevant resistance in Gram-negative bacteria. RND transporters are found in representatives of all kingdoms. However, homology between bacterial and human proteins is negligibly low [55]. In bacteria these pumps are organized in complex three component structures, which traverse both the inner and outer bacterial membranes. They consist of a transporter located in the cytoplasmic membrane, an outer membrane channel in the outer membrane and a periplasmic 'linker' protein, which brings the other two components into contact. This structural organization allows extrusion of substrates from the cytoplasmic and periplasmic compartments directly into the external medium, thus taking advantage of the accelerated uptake through the outer membrane [56,57].

Studies on the structural biology of these tripartite pumps have enjoyed enormous and unprecedented success. The high-resolution 3D-structures of all three individual components are now available. The structure of the *E. coli* outer membrane protein (OMP) TolC was solved in 2000 [58]. 2002 was marked by the elucidation of the structure of the *E. coli* RND protein AcrB [59], rapidly followed by the structure of same protein in combination with several ligands in 2003 [51]. Finally, 2004 heralded the structure of the last constituent, the membrane fusion protein (MFP) component, MexA from *P. aeruginosa* [60,61] as well as the structure of the *P. aeruginosa* OMP, OprM protein [62].

X-ray crystallography provides a strong structural basis in support of the concept of trans-envelope efflux. Both homotrimeric AcrB and TolC proteins contain large periplasmic domains. There appears to be a perfect fit between the funnel-like opening of the headpiece of AcrB and the proximal end of the tunnel-like TolC. Thus the deep 'crater' at the tip of the periplasmic segment of AcrB may serve as a portal connecting AcrB to the TolC subunit. The latter likely serves as an 'exhaust pipe' conducting the substrates expelled by the pump through the outer membrane and into the extracellular space [63,64]. As for the MFP, it is hypothesized that this protein is required for stable association of the inner and outer membrane components of the pump [60,61,65–69]. The 3D-structure of the MFP MexA protein is compatible with this interpretation.

The 3D-architecture of the AcrB trimer reveals three large vestibules, formed by neighboring protomers, wide-open to the periplasm. These vestibules lead to a central cavity inside the pump. It has been hypothesized that this spacious cavity serves as a binding site for multiple substrates, which gain access to the pump from the periplasmic space. Indeed, in later co-crystallization studies several structurally unrelated substrates of AcrB were localized to the central cavity [51,70]. It is also quite possible that alternative or additional binding site(s) exist between the periplasmic domains. This possibility is supported experimentally based on domain swapping experiments and the location of mutations affecting substrate specificity [69,71–75].

Several laboratories are working extensively on improving resolution of the structure (currently, at 3.5 Å), which should open a unique opportunity to apply a protein structure-based approach to both the discovery and optimization of efflux pump inhibitors (EPIs), possibly also aiding in the design of antibiotics with decreased propensity for recognition by the transporter [76]. While undoubtedly useful and likely facilitating in the future, structural elucidation of these pumps is not absolutely required in order to search for and to optimize efflux pump inhibitors for clinical use. Primary and secondary high-throughput assays are readily available for lead discovery, and several inhibitors of RND transporters have been reported in the literature (see below). The lack of close human homologs, the simplicity of screening, the availability of robust secondary assays, the extracytoplasmic-binding sites and the proven drugability of existing leads make MDR RND transporters perfect discovery targets.

3. Validation of the EPI strategy

In the late 1990s, Microcide and Daiichi Pharmaceuticals undertook a comprehensive program to search for and develop EPIs for Gram-negative bacteria. The specific goal of their collaborative program was to potentiate the activity of levofloxacin, a substrate for multiple homologous tripartite multidrug resistance pumps belonging to the RND-family of transporters. Four of these tripartite pumps in *P. aeruginosa* (MexAB–OprM, MexCD–OprJ, MexEF–OprN and MexXY–OprM) are capable of conferring clinical resistance to this antibiotic [18]. A simple screening strategy for selection of antibiotic

potentiators was used to identify EPIs. Importantly, when this strategy was applied to *P. aeruginosa*, it also turned up compounds capable of permeabilizing the outer membrane, stressing the importance of the follow-up secondary assays [77,104]. The first identified efflux pump inhibitor, MC-207,110 [77], effectively inhibits all four clinically relevant *P. aeruginosa* pumps as well as similar RND pumps from other Gram-negative bacteria. Based on the broad spectrum of pump inhibition in various Gram-negative bacteria, compounds in this drug class are considered broad-spectrum EPIs. Interestingly, not all antibiotic substrates for a given pump are potentiated by MC-207,110. The degree of pump inhibition is dependent on the nature of the substrate. For example, MC-207,110 potentiates fluoroquinolones, macrolides/ketolides, oxazolidinones, chloramphenicol and rifampicin, but not β-lactams or aminoglycosides. Mechanism of action studies indicated that MC-207,110 itself is a substrate of efflux pumps [78]. The assumption is that different antibiotics have non-identical binding pockets within the transporter protein and that MC-207,110 works by competing with antibiotics for binding in the substrate pocket specific to the potentiated antibiotic, but not to the binding site for the non-potentiated antibiotics, explaining the substrate-dependent inhibition. This may also explain why attempts to isolate target-based mutations conferring resistance to MC-207,110 (making the efflux pump non-susceptible to inhibition) were unsuccessful [Lomovskaya, unpublished results]. Most likely, such mutations would render the pump incapable of interacting with other substrates and hence be observed as inactive. This being the case, specific targeting of the pump substrate-binding site may be a viable future strategy to design alternative or improved efflux pump inhibitors. It also became clear during the course of the program that in any empiric search for efflux pump inhibitors it is very important to identify and use specific partner antibiotics.

MC-207,110 decreased intrinsic resistance to levofloxacin about 8-fold in wild-type strains of *P. aeruginosa*, while efflux pump overexpressing strain susceptibility may be increased up to 64-fold. This same degree of potentiation is observed irrespective of the presence of target-based mutations in DNA gyrase or DNA topoisomerase IV. Recent clinical isolates of *P. aeruginosa* with a wide range of resistant phenotypes also showed increased susceptibility to levofloxacin in the presence of MC-207,110. Remarkably, both the MIC_{50} and the MIC_{90} were decreased to the same extent, 16-fold by MC-207,110, providing additional evidence that the potentiating effect is not dependent on the absolute level of resistance, but solely on the level of efflux pump expression. In the presence of the inhibitor, both MIC_{50} and MIC_{90} were below the susceptibility barrier for levofloxacin.

Of particular importance is the observation that the selection frequency for fluoroquinolone resistant bacteria was also dramatically decreased in the presence of MC-207,110. The appearance of both efflux-mediated and target-based mutations is minimized. This is presumably because the inhibitor decreases MexAB–OprM-mediated intrinsic resistance to the level at which a single target-based mutation does not confer enough resistance to emerge under selection conditions [77]. Suppression of resistance development was also demonstrated in vivo using later stage compounds, in the neutropenic mouse thigh model of *P. aeruginosa* infection [79,80].

The attractiveness of MC-207,110 as a lead was based on its broad-spectrum efflux pump inhibitory activity. Such broad-spectrum activity is absolutely needed in order to have a clinically significant impact on fluoroquinolones, which are extruded by multiple efflux pumps. However, broad-spectrum EPI activity is not always essential. For example, resistance to aminoglycosides is conferred by a single pump, MexXY–OprM [81], while it is mainly MexAB–OprM, which confers resistance to β-lactams in *P. aeruginosa* [49,82].

EPIs with high selectivity towards MexAB–OprM were also identified in the Microcide–Daiichi collaboration [83–86]. One such series of compounds, the pyridopyrimidines, unlike MC-207,110 inhibit the efflux of all substrates of the MexAB–OprM pump, including β-lactams and fluoroquinolones, with little effect on the other Mex systems. In later studies, mutagen induced mutations conferring resistance to potentiation by these compounds were identified in the MexB gene [Lomovskaya, unpublished results]. Of note is that these mutations were not cross-resistant to MC-207,110, confirming the differences in modes of action of these two types of EPIs. Resistance studies demonstrated that while mutants with simultaneous resistance to β-lactams and fluoroquinilones due to overexpression of MexAB–OprM could be easily isolated, no such mutants were selected in the presence of MexAB–OprM-selective EPIs [Lomovskaya, unpublished results].

These studies demonstrated that multiple inhibitors of a single pump could be identified. They also validated the belief that inhibition of efflux pumps is a viable strategy to reversing antibiotic resistance and blocking its development [87–90]. Extensive efforts have been made to improve the potency and the *(ADMET) profile of MC-207,100 series of compounds [79,80,91,92]. It has two basic moieties that were shown to be essential for activity. Unfortunately, the same moieties were found to be associated with unfavorable pharmacokinetic and toxicological profiles, and development of this lead series was suspended. While downgraded at least temporarily from drug candidate status, these compounds are widely used as a research tool, owing to broad-spectrum EPI activity, to evaluate the contribution of efflux pumps to antibiotic resistance in clinical isolates of *P. aeruginosa* and other Gram-negative bacteria [93–101].

Several other structural classes of inhibitors of the RND transporters are described in the literature, though none have been reported to have EPI activity against efflux pumps from *P. aeruginosa*. In a high-throughput screening assay for potentiators of novobiocin, scientists at Pharmacia, now Pfizer, identified 3-arylpiperidines as inhibitors of the AcrAB–TolC pump from *E. coli* [102]. In a report from University Hospital in Freiburg, Germany, selected arylpirerazines have been shown to inhibit the same transporter and to potentiate activity of its multiple antibiotic substrates [103]. Several laboratories in France undertook systematic efforts to identify and characterize various alkoxy- and alkylaminoquinolines as EPIs showing significant activity against laboratory and clinical strains of *Enterobacter aerogenes* and *Klebsiella pneumonia* [104–107]. To the authors' knowledge, no large-scale development programs have been initiated based on these compounds.

4. Challenges and perspectives

Many challenges are encountered on the path to conversion of a drug lead with an attractive new mode of action into a clinically useful therapeutic agent. In this regard, an efflux pump inhibitor is no different from any other new chemical entity (NCE). As well-appreciated by drug hunters, more risk is associated with the development of an NCE than an improved representative from an already widely used and proven class of antibiotics (with combination of clinical benefits and appropriate toxicological profile validated by thousands of patients), hence, the continued efforts to develop yet more improved β-lactams, fluoroquinolones, macrolides and glycopeptides. However, what if an efflux pump inhibitor is found that is an existing drug already used clinically for a different indication by a different mode of action? Provided that its EPI activity is potent enough to not require significantly higher doses than already established clinically (ensuring no danger to uncover unknown toxicities), developing it as an EPI would mitigate the NCE-imposed risk.

Additional challenges are associated with the fact that antimicrobial therapy with efflux pump inhibitors is a combination therapy by its very nature. In order to provide the maximum pharmacodynamic benefit, the pharmacokinetics of an EPI should be appropriately tailored to pharmacokinetics of the antibiotic component of the combination. This does not mean that pharmacokinetic profiles for both agents should be identical. Let us imagine the following combination: antibiotic component is a fluoroquinolone or an aminoglycoside, while the efflux pump inhibitor is a compound with the mode of action similar to that described above for MC-207,110. Both antibiotics are concentration-dependent drugs with an efficacy driven by Peak/MIC (and AUC/MIC) ratios [108,109]. At the same time, the EPI is a competitive inhibitor and its pharmacodynamic effect will most probably be driven by the time its concentration in the body exceeds the concentration associated with IC_{50} of pump inhibition. Hence, no need to match, but rather appropriately tailor the pharmacokinetics to maximize the synergy between the two agents. This pharmacokinetic tailoring to establish the optimal ratio and dosing regimens to create the best possible efficacy while maintaining appropriate toxicological profiles, represents a non-arguable challenge. On the positive side is the fact that animal models with engineered strains lacking efflux pumps can be used to very precisely define the pharmacokinetic/pharmacodynamic targets associated with the best impact for the EPIs on the efficacy of the partner antibiotics.

Additional challenges lay in the design of clinical trials and are of a regulatory character. For example, the major beneficial consequence of combining an EPI with a fluoroquinolone is a reduction in rates of resistance development, which is best demonstrated by bacteriologic endpoints and using PK/PD in vitro and animal models of infection [110–114]. However, it is clinical endpoints, which are relevant in the FDA approval process. Some changes in regulatory decision-making regarding resistance would be very helpful in order to facilitate approval based on prevention of resistance development. Another important benefit provided by EPIs is actual reversing of resistance. To demonstrate this benefit, large clinical trials would need to be performed in order to enroll sufficient amount of patients carrying resistant strains. This problem might be mitigated if the results of preclinical in vitro and in vivo PK/PD studies might be used to demonstrate that a drug has similar activity against strains that are susceptible or resistant to an antibiotic component of the combination. Based on availability of these results clinical data against susceptible strains may be supportive of efficacy against resistant strains (although some clinical data against resistant strains still will be necessary). It appears that many significant innovations are being considered by FDA to facilitate and stimulate the development of new antibiotics (www.fda.gov/cder/drug/antimicrobial/FDA_IDSA_ISAP_Presentations.htm).

Where does industry stand with regard to developing efflux pump inhibitors? There is indisputably high awareness of the contribution of efflux to both intrinsic and acquired antibiotic resistance as well as practical attention given to the implications for antibiotic development. In fact, most researchers involved in antibacterial drug discovery and development routinely evaluate the impact of efflux pumps on their ''favorite'' compounds, using either strains lacking efflux pumps or broad-spectrum efflux inhibitors, such as MC-207,110. Despite such appreciation, to our knowledge, Mpex Pharmaceuticals (the affiliation for both authors of this article), is the only biopharmaceutical company currently committed to discovering and developing small molecule inhibitors of RND efflux pumps from Gram-negative bacteria to overcome resistance and improve clinical outcomes of antibiotic treatment of recalcitrant infections, such as those associated with cystic fibrosis and other nosocomial respiratory diseases. In fact, Phase I clinical trials for the first efflux pump inhibitor development candidate, MP-601,205, have been initiated. In addition, Mpex scientists are working on other combination antibacterials for the treatment of resistant bacterial infections in the hospital and community settings. Bacteria use efflux pumps to become resistant to antibiotics. This alone makes pumps worth inhibiting. However, it is the successful introduction of efflux pump inhibitors in clinical practice and demonstration of their multifactorial benefits that will provide the ultimate validation of the EPI-based combination approach.

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