Review Article

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Efflux pump inhibitors for bacterial pathogens: From bench to bedside

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With the advent of antibiotics, bacterial infections were supposed to be a thing of past. However, this instead led to the selection and evolution of bacteria with mechanisms to counter the action of antibiotics. Antibiotic efflux is one of the major mechanisms, whereby bacteria pump out the antibiotics from their cellular interior to the external environment using special transporter proteins called efflux pumps. Inhibiting these pumps seems to be an attractive strategy at a time when novel antibiotic supplies are dwindling. Molecules capable of inhibiting these pumps, known as efflux pump inhibitors (EPIs), have been viewed as potential therapeutic agents that can rejuvenate the activity of antibiotics that are no longer effective against bacterial pathogens. EPIs follow some general mechanisms of efflux inhibition and are derived from various natural as well as synthetic sources. This review focuses on EPIs and identifies the challenges that have kept these futuristic therapeutics away from the commercial realm so far.

Key words Antibiotics - efflux pumps - multiple drug resistance - pathogens - therapeutics

Introduction

With the discovery of penicillin and streptomycin in the early 20th century, we entered the antibiotic era where previously considered deadly bacterial infections could be easily treated. The mid-decades of the 20th century witnessed the 'golden age' of antibiotic discovery as about half of the antibiotics in use today were discovered during that period[1]. However, the rampant use, misuse and abuse of antibiotics accelerated the evolution of bacteria, resulting in selection of antibiotic-resistant bacteria[2]. The Centers for Disease Control and Prevention (CDC), USA, estimate states that about 30 per cent of the antibiotics prescribed to the outpatients are unnecessary[3]. The reckless use of broad-spectrum antibiotics as growth promoters in animal farming has also aggravated the problem. The gravity of the situation can be understood by the fact that in a developed nation like the USA, nearly two million people develop hospital-acquired infections from drug-resistant bacteria that leave about a hundred thousand dead[4]. Estimates on medical expense per patient with antibiotic-resistant infections vary from $18,588 to $29,069 which ultimately amounts to a healthcare loss as high as $20 billion and a productivity loss of $35 billion every year[5]. The situation is much worse in economically backward countries that are generally plagued by poor sanitary, health and medical conditions. The increasing incidence of multidrug-resistant (MDR),

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extensively drug-resistant (XDR, resistant to all but one or two classes of antibiotics) and pan-drug-resistant (PDR, resistant to all classes of antibiotics) microbes has brought us to the brink of the 'post-antibiotic era' where no antibiotics will be effective any longer and even slightest of infections would prove deadly[6]. The serious threat this situation poses was recognized by the UN General Assembly that drew a framework for all the nations to co-operate and work in the direction of combating antimicrobial resistance[7].

Bacteria develop resistance to antibiotics through four major mechanisms (Fig. 1): (*i*) altering the cellular permeability to avoid the entry of antibiotics into the cells, (*ii*) modifying the molecular targets of the antibiotics so that they can no longer act on them, (*iii*) enzymatic modification of antibiotics to render them inactive, and (*iv*) expression of efflux pumps to pump out antibiotics from the cellular milieu[8]. These factors responsible for resistance could be intrinsic or acquired through various mechanisms. The presence of determinants of resistance on mobile genetic elements such as plasmids and transposons combined with the free mobility of human carriers has resulted in dissemination of drug resistance to a wide variety of bacterial genera and geographical locations. The research endeavour that previously had the sole aim of discovery of novel antibiotics now has added burden of understanding the development of resistance and strategies to reverse them. This review focuses on one of the causes of antibiotic resistance,

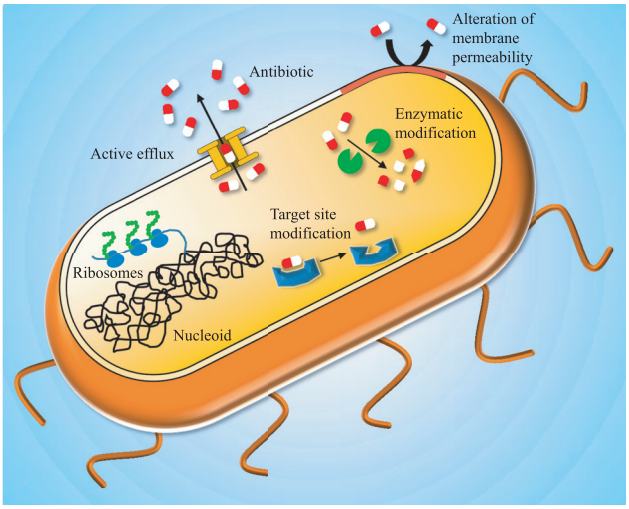

Fig. 1. The four major mechanisms by which the bacterial cells develop multiple drug resistance. (*i*) Altering the cellular permeability to avoid the entry of antibiotics into the cells, (*ii*) modifying the targets of the antibiotics so that they can no longer act on them, (*iii*) enzymatic modification of antibiotics to render them inactive, and (*iv*) expression of efflux pumps to pump out antibiotics from cell interior.

the efflux pumps, and deals with the current progress in inhibiting these determinants of resistance.

Bacterial efflux systems as determinants of multidrug resistance

Efflux pumps are bacterial transport proteins which are involved in extrusion of substrates from the cellular interior to the external environment. These substrates are often antibiotics, imparting the efflux pump expressing bacteria antibiotic resistant phenotype[9]. From the first drug-resistant efflux pump discovered in the 1990s, the development in molecular microbiology has led to the characterization of many efflux pumps in Gram-positive bacteria (GPB) including methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumoniae*, *Clostridium difficile*, *Enterococcus* spp. and *Listeria monocytogenes* and Gram-negative bacteria (GNB) such as *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Vibrio cholerae* and *Salmonella* spp.[10,11]. Since these transport substrates against a concentration gradient, these efflux pumps are energy dependent. Based on the mechanism by which these derive this energy, the efflux pumps are broadly classified into two categories. The primary efflux pumps draw energy from active hydrolysis of ATP, whereas the secondary efflux pumps draw energy from chemical gradients formed by either protons or ions such as sodium. Five major families of efflux pumps have been described in the prokaryotes (Fig. 2), namely (*i*) ATP binding cassette (ABC), which are primary active transporters, (*ii*) small multidrug resistance family, (*iii*) multidrug and toxin extrusion (MATE) family, (*iv*) major facilitator superfamily (MFS) and (*v*) resistance nodulation cell division (RND) family, which are all secondary active transporters[12]. The complexity with which these pump proteins are organized has also provided insight into their structure and molecular mechanism of substrate transport. The drug resistance in GPB is mainly mediated by cytoplasmic membrane located efflux transporters, while the efflux pumps in GNB are more complex due to their multi-layered cell envelop: the inner or cytoplasmic membrane and the outer membrane, which are separated by the periplasmic space that combines to form a tripartite protein channel through which the drug is effluxed. RND family efflux pumps have tripartite organization and are the major contributors to intrinsic antibiotic resistance in GNB, which expel a broad spectrum of antibiotics and biocides, including

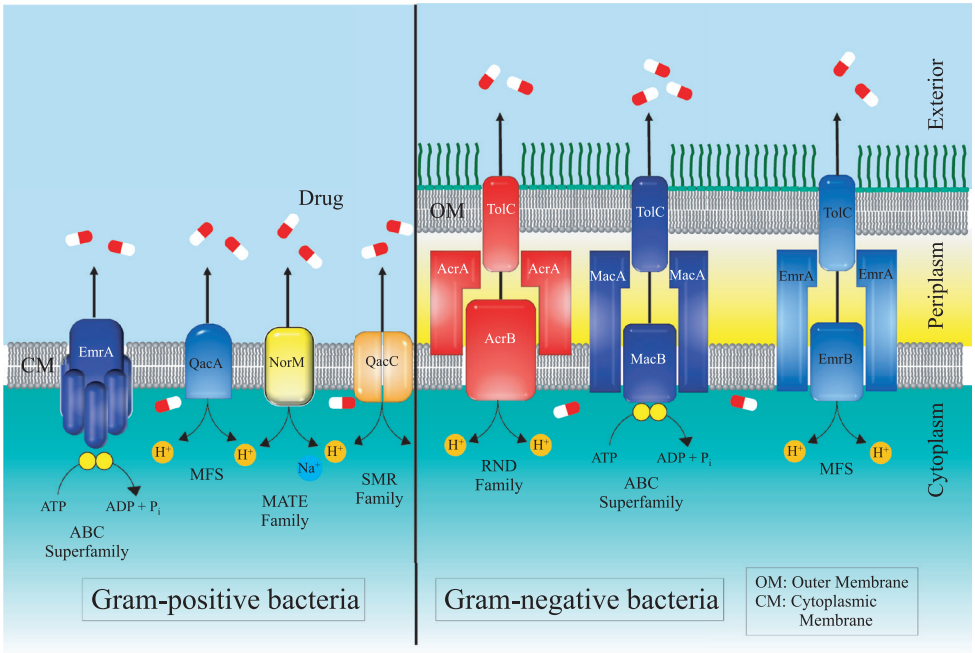

Fig. 2. The five classes of efflux pumps in bacteria, (*i*) ATP-binding cassette superfamily, (*ii*) major facilitator superfamily, (*iii*) multidrug and toxic compound extrusion family, (*iv*) small multidrug resistance family, and (*v*) resistance nodulation division family. The organization of these efflux pumps is different in Gram-positive and Gram-negative bacteria.

fluoroquinolones, β-lactams, tetracycline and linezolid. However, in GPB, MFS transporters are predominant including NorA of *S. aureus*, PmrA of *S. pneumoniae* and EmeA of *E. faecalis* that extrude a large number of antibiotics belonging to different classes[10,11].

Efflux pumps, unlike most other determinants of resistance, are more often intrinsic. The genes coding for these transporters are found in susceptible as well as resistant bacteria[13] and are often parts of an operon whose expression is regulated at the transcriptional level. The mutations in the regulatory proteins or the mutations at the promoters result in overexpression of these efflux pumps, resulting in drug resistance[13]. Bacterial efflux system can be either specific, extruding only one or a single class of antibiotics (such as TetA and AbaF that selectively exclude specific antibiotics such as tetracycline and fosfomycin, respectively)[14] or capable of pumping out several classes of antibiotics (such as MexAB-OprM, NorA and BmrA that extrude distinct class of antibiotics, disinfectant, dyes and detergents) being designated as MDR efflux pumps. Most of the MDR efflux pumps are chromosomally encoded including NorA, NorB, MepA and MdeA of *S. aureus* that are responsible for intrinsic resistance in bacteria to several antibiotics, while some of the pumps are encoded on plasmids (QacA/B of *S. aureus*) or transposons (MefA and MefB of *Streptococcus* spp.) that provide the transferable mode of resistance[15,16].

Apart from drug resistance, the physiological role of efflux pumps in bacteria extends to bile tolerance in enteric bacteria, leading to colonization, increase in virulence, biofilm secretion and bacterial survival in the host[17].

Efflux pump inhibitors as new therapeutic agents

Considering the importance of efflux in mediating antibiotic resistance, it is worthwhile to expect that circumventing these determinants of resistance could potentiate the activity of substrate antibiotics. Abolishment the efflux could be achieved by different ways namely, (*i*) downregulating the expression of efflux pump genes by interfering in genetic regulation, (*ii*) redesigning antibiotics that are no longer recognized as substrates, (*iii*) inhibiting the assembly of functional efflux pumps, (*iv*) blocking the pump to avoid substrate binding to the active site, and (*v*) collapsing the energy mechanism responsible for energizing these pumps[18]. This review mainly focuses on the last two categories that attempt to inhibit the efflux pumps using chemical entities called efflux pump inhibitors (EPIs). EPIs are the molecules that inhibit efflux pumps by one or more mechanisms, leading to inactive drug transport. Since this could eventually lead to successful build-

up of an antibiotic inside the cell, these EPIs can be used as adjuncts in combination with antibiotics to enhance their activity against bacteria expressing efflux pumps. The possibility of using EPIs to rejuvenate the activity of antibiotics has been at an experimental stage since the beginning of this century. MC-207,110 [phenylalanyl arginyl β-naphthylamide (PAβN)], a peptidomimetic EPI, was the first to be discovered in 2001. It potentiates the antibacterial activity of levofloxacin and erythromycin against MexAB-OprM-overexpressing clinical isolates of *P. aeruginosa*[19]. However, the success has been limited, and no EPI has made it to the commercial realm so far.

A chemical entity would have to go through a stringent checklist to make it as a successful EPI. First, the molecule must not be antibacterial *per se*. An antibacterial molecule would ultimately lead to selection of mutants resistant to its action that will severely impact its utility as an EPI. Second, the molecule should be selective and not target any eukaryotic efflux pumps. Since efflux pumps are ubiquitous and their basic functional aspects tend to be similar across the life forms, selective inhibition of bacterial efflux pumps becomes a difficult task. Third, it should possess ideal pharmacological features such as non-toxicity, high therapeutic and safety indices, good ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) profile and serum stability. Finally, to be successful at a commercial level, the production of the EPI must be economically feasible[18].

Types of EPIs based on their mechanism of action

The EPIs in laboratories have shown good promise as therapeutic adjuvants. Although a multitude of EPIs have been reported with different modes of action, these can be broadly characterized into two categories:

Energy dissipation

Since efflux pumps are dependent on cellular energy, the decoupling of the energy and efflux activity presents an interesting approach to efflux inhibition. The proton gradient or the ATPase that supplies energy to these pumps has been tried as targets of various EPIs. Such an inhibition scheme does not require any direct interaction of the inhibitor with the efflux pump itself. This approach appears to be advantageous as many efflux pumps are dependent on the proton gradient, making this a universal scheme for inhibiting them.

Carbonyl cyanide-m-chlorophenylhydrazone (CCCP) is perhaps the most well-known laboratory EPI. It is an ionophore that disrupts the proton motive force (PMF) by affecting both its components, $\Delta\psi$ and ΔpH[20]. This also makes the bacterial cells metabolically inactive giving rise to the debate whether the synergistic effect that CCCP shows with a range of antibiotics is actually a consequence of efflux pump inactivity or metabolic inactivity of the cells. The CCCP has been reported to revive the activity of tetracycline in *Helicobacter pylori* and *Klebsiella* spp.[21,22]. Synergy between carbapenems and CCCP was also reported, which was independent of the efflux inhibition activity of CCCP, supporting the previous hypothesis that CCCP leads to metabolically inactive cells giving rise to synergistic effect with antibiotics[23]. This combined with the cellular toxicity towards mammalian cells has kept CCCP limited to laboratory use only.

Our group has also reported a synthetic EPI, IITR08027, from a library of 8000 synthetic molecules that was screened for potentiators of ciprofloxacin[20]. The molecule was found to be very effective at reversing the resistance against fluoroquinolones in both recombinant *E. coli* and clinical strains of *A. baumannii* overexpressing the MATE efflux pump AbeM. IITR08027 disturbs the proton gradient that is necessary for energizing the pump. Since it had a little impact on the $\Delta\psi$ component of PMF, it did not have any antibacterial effect of its own and displayed low toxicity towards animal cells. These qualities of IITR08027 make it very close to an ideal EPI and it is being assessed for its preclinical potential.

Inhibition by direct binding

Another mechanism of efflux pump inhibition is the binding of the EPIs to functional efflux pumps, resulting in reduced ability of the pumps to interact with their substrates. This binding could be competitive, where the EPI competes with the substrates for the same binding site; or non-competitive, where the binding of EPI to the pump causes decrease in the affinity of pump towards its substrates. However, bacteria can always mutate their efflux pumps to modify the target sites of these inhibitors, rendering them useless.

PAβN (or MC-207,110) is a paradigm in synthetic EPIs as it was the first inhibitor of the RND family pumps. Screened from a synthetic library as a potentiator of levofloxacin against *P. aeruginosa* cells expressing MexAB, MexCD and MexEF pumps, this molecule also potentiates erythromycin and chloramphenicol[19]. Since it is a substrate for the RND pumps as well, it acts as a competitive inhibitor of substrate binding and efflux.

PAβN is not as effective when combined with tetracycline and carbenicillin, suggesting that these antibiotics have a binding site different from that of PAβN. There is not much scientific evidence about the mechanism of action of PAβN, but computational simulations with AcrB have predicted that it interacts with F_{135}, F_{178}, F_{615}, F_{628}, Q_{176} and E_{673} residues[24]. Although there is some degree of evidence that it additionally affects the outer membrane permeability, there is a requirement of more investigations to lay a strong claim[19].

Verapamil is a small molecule that acts as an ion channel blocker and is used in the treatment of hypertension. Studies in *Mycobacterium tuberculosis* have shown that verapamil potentiates the activity of bedaquiline and ofloxacin[25,26]. Further studies have identified that verapamil inhibits the activity of MATE pumps. It has a low amount of toxicity towards bacterial cells not expressing MATE efflux pumps, suggesting specificity towards bacteria expressing these pumps and a competitive mode of inhibition. Crystallization studies confirmed that verapamil binds to the active site of the MATE efflux pumps in a manner similar to the substrates of the pump. Although verapamil interacted with two prototype MATE pumps, DinF and NorM, in a separate manner, the overall effect of inhibition of pump activity was the same[27].

Another molecule conforming to this category, 1-(1-napthylmethyl)-piperazine (NMP), was derived from a parent molecule that was screened out of a synthetic library of compounds[28]. The library was assessed for potentiators of levofloxacin in *E. coli* cells overexpressing the efflux pumps AcrAB and AcrEF. NMP resulted in increased accumulation of levofloxacin in the cells, which enhanced its activity. NMP was also found to potentiate oxacillin, rifampin, chloramphenicol and clarithromycin and to lower extent fluoroquinolones, azithromycin, clindamycin, nitrofurantoin and doxycycline[28]. Mutagenesis using error-prone PCR resulted in AcrB mutants resistant to the potentiating activity of NMP. This resulted in the identification of core residues, G_{141}, N_{282} and F_{610}, which are crucial for NMP binding. NMP interacts with the F_{610}, residue and causes conformational change in AcrB, resulting in inhibition in a non-competitive manner[29]. However, the molecule also has an antibacterial property at a concentration four-fold higher than what is used as an EPI, suggesting a secondary target for the molecule as well.

Types of EPIs based on their origin

Although many molecules have shown potential as EPIs, the mechanism of action is not known for a majority of them. Therefore, it becomes difficult to fit such molecules in a classification scheme based on their mode of action. To accommodate EPIs with no definite mode of action, the EPIs can be categorized based on their source. This leads to three broad categories that include EPIs derived from plant products, synthetic chemistry and microorganisms.

Plant-derived EPIs

Plant-derived phytochemicals include a wide variety of chemical adjuvants that synergistically enhance the efficacy of antibiotics up to several folds[30]. Major subclasses of plant-derived EPIs are enumerated as follows:

Plant alkaloids: Reserpine, an antipsychotic drug extracted from the roots of *Rauwolfia serpentina*, is a promising EPI that targets efflux pumps of the MFS and RND superfamily[30]. Reserpine is reported to potentiate antimicrobial activity of antibiotics by interacting directly with amino acid residues in the efflux transporter protein Bmr, which mediates tetracycline efflux in *B. subtilis*. In addition, reserpine has also been shown to reverse NorA-mediated resistance in *S. aureus* by enhancing the activity of norfloxacin up to four-fold[31]. The clinical application of reserpine with clinically used antibiotics, however, has not yet been achieved due to its nephrotoxic nature[32].

Piperine (isolated from *Piper nigram*) is another alkaloid known to inhibit the human P-glycoprotein of ABC transporters via cytochrome P450-mediated pathways. The efflux pump inhibitory activity of both piperine and its derivative, piperidine, has also been reported against pathogenic bacteria including *S. aureus* and *Mycobacteria* spp.[33]. A study conducted in *S. aureus* showed that piperine enhances the accumulation of ciprofloxacin by inhibiting NorA efflux pump. In *M. tuberculosis H37Rv* and several clinical isolates, piperine has been reported to potentiate the activity of rifampicin by inhibiting an uncharacterized efflux pump – Rv1258c. In *Mycobacterium smegmatis,* piperine has been shown to decrease the MIC of ethidium bromide indicating its application as an EPI across bacterial genera[34].

Flavonoids: Baicalein, a 5,6,7-trihydroflavone, is a weak antimicrobial flavone isolated from thyme leaves (*Thymus vulgaris*). It improves the susceptibility

of clinical MRSA strain towards ciprofloxacin and β-lactam antibiotics including oxacillin, cefmetazole and ampicillin[35,36]. Baicalein is also reported to increase the potency of tetracycline in TetK-overexpressing *Staphylococci* by inhibiting the uptake of [^{3}H] tetracycline[36].

5'-methoxy-hydnocarpin, a flavolignan isolated from *Berberis fremontii*, has been reported to enhance the efficacy of several NorA substrates, including norfloxacin and berberine by inhibiting this proton pump. However, due to its toxic nature, its clinical success is doubtful[37]. Some of the other plant derived isoflavones (isolated from *Lupinus argenteus*) including genistein, orobol and biochanin A, have been reported to reduce the MIC of berberine and norfloxacin in clinical *S. aureus* and *M. smegmatis* by blocking the MDR efflux pumps[38].

Polyphenols: Catechin gallates, a group of phenolic metabolites, have been reported to reverse the MRSA resistance. Catechin gallates such as epicatechin gallate and epigallocatechin gallate are weak inhibitors of NorA efflux pump, with epicatechin gallate being slightly more potent. Interestingly, both compounds have been reported to enhance the efflux at low concentrations[39]. It has been proposed that these molecules have two different binding sites on the NorA efflux transporter with different affinities. At low concentrations, catechins occupy high-affinity binding sites leading to increased efflux of NorA substrate. Their effect as EPI is observed only at a higher concentration.

Epigallocatechin gallate has also been reported to enhance the potency of tetracycline, erythromycin and ciprofloxacin in TetK-overexpressing Gram-positive *Staphylococci* and in Gram-negative *Campylobacter* spp. However, due to toxicity concerns associated with it, further *in vivo* and pre-clinical studies were not undertaken[40].

Phenolic diterpenes: Phenolic diterpenes, such as carnosic acid and carnosol, isolated from herb Rosemary (*Rosmarinus officinalis*), have been reported as EPIs. These enhance the potency of antibiotics such as tetracycline and erythromycin against macrolide-resistant strain of *S. aureus* expressing the ABC transporter MsrA and TetK efflux pumps[41].

Geraniol (monoterpenoid alcohol), isolated from *Helichrysum italicum*, has also been reported to modulate drug resistance in several GNB species by targeting MDR efflux mechanisms. It decreases the MIC of chloramphenicol in *Enterobacter aerogenes* CM-64 strain that overexpresses the tripartite efflux pump, AcrAB-TolC[42].

EPIs of synthetic origin

Apart from natural plant-derived products, screening of novel semi-synthetic or synthetic diversified chemical libraries is a useful way to identify potential EPIs. Many screening efforts have yielded results with varying amount of success. Such synthetic small molecule EPIs can be further classified as follows:

Peptidomimetic compounds: The dipeptide amide compound PAβN was one of the first EPIs discovered through chemical genetics approach. It has been reported to potentiate the activity of many antibiotics including fluoroquinolones, macrolides and chloramphenicol in GNB by inhibiting RND efflux pumps[19,24]. However, it had limited clinical potential due to toxicity towards mammalian cells. Although some synthetic derivatives with different basic properties such as reduced toxicity, enhanced stability, and better solubility were evaluated, none of the active analogues could significantly reduce the drawback of the parent molecule. Thus, PAβN and its novel derivatives are limited to use in laboratory as standards to determine the level of inhibitor-sensitive efflux for specific antibiotics in various bacterial pathogens[43].

Quinoline derivatives: This novel class of compounds was discovered by using several screening approaches against clinical MDR bacterial strains. Quinoline derivatives such as pyridoquinolones can restore the activity of norfloxacin in *E. aerogenes* overexpressing the AcrAB-TolC efflux pump, by acting as competitive inhibitor of this RND pump[44]. Some other synthetic analogues such as 4-substituted thioalkyl, alkylamino and alkoxy quinolone have also been reported to enhance the activity of tetracyclines, norfloxacin and chloramphenicol in clinical isolates of *K. pneumoniae* and *E. aerogenes*[45]. A series of 2-phenyl-4(1H)-quinolone and 2-phenyl-4-hydroxyquinoline derivatives have been synthesized by modifying the flavone scaffold and these have been reported as potent inhibitors of NorA efflux pump in *S. aureus*[46].

Arylpiperidines and aryl piperazine derivatives: Arylpiperidine and its derivatives such as 3-arylpiperidine have been reported to restore susceptibility to linezolid and enhance its accumulation in *E. coli*[47]. Another series of analogues, phenylpiperidines, which are selective serotonin re-uptake inhibitors, are known to inhibit the function of

S. aureus MDR efflux pumps. These compounds also affect the activity of the AcrAB-TolC pump in *E. coli* partially but have no effect on the efflux activity of the *P. aeruginosa* RND efflux pumps such as MexAB-OprM or MexCD-OprJ[48].

One of the leading arylpiperazine compounds, NMP, has been shown to restore the activity of RND pump substrates including levofloxacin and EtBr in *E. coli*-overexpressing AcrAB and AcrEF. However, due to serotonin re-uptake inhibitor property of arylpiperazines, these compounds are likely to be toxic to mammalian cells[28].

Pyridopyrimidine and pyranopyridine derivatives: Pyridopyrimidine analogues D2 and D13-9001 have been reported as MexAB-OprM-specific pump inhibitor in MexAB overexpressing *P. aeruginosa* under both *in vitro* and *in vivo* conditions[49]. It has been proposed that D13-9001 is able to inhibit the efflux of antibiotics by binding to specific site in efflux pumps (AcrB in *E. coli* and MexB in *P. aeruginosa*). Further, the crystallographic data suggested that the hydrophobic tert-butyl thiazolyl aminocarboxyl moiety of D13-9001 binds tightly to the hydrophobic trap in deep substrate binding pocket of the pump and prevents the conformational changes that are needed for the proper activity of the pump. In addition, the hydrophilic component of D13-9001 is also reported to interact with the substrate binding channel of pump, thereby preventing the substrate binding to the pumps[43].

MBX2319, a synthetic pyrazolopyridine, was screened as a potentiator of fluoroquinolones antibiotics from a library of small molecules. It enhances the efficacy of ciprofloxacin, levofloxacin and piperacillin up to eight-fold against *E. coli AB1157*[29]. Further, MBX2319 also led to increased intracellular accumulation of Hoechst dye in wild type and AcrAB-TolC-overexpressing *E. coli*[29]. A detailed X-ray crystallographic study suggested that MBX2319 interacts with the hydrophobic trap of the AcrB pump with its pyridine ring predicted to form a ring stacking interaction with the amino acid residues[43].

In addition, many synthetic/semisynthetic derivatives have been synthesized artificially that mainly target MDR efflux pump of both GPB and GNB (Table).

EPIs derived from microbes

Although most of the EPIs have their origin in natural products or semi-synthetic/synthetic chemical libraries, a small fraction of EPIs has been reported to originate from microbes. EA-371α and EA-371δ, first extracted from fermentation extract of *Streptomyces* spp., have been recognized as specific inhibitors of the MexAB-OprM pump in *P. Aeruginosa*[101]. The novel structure of these compounds offers an opportunity to the researchers to synthesize novel derivatives with increased potency, bioavailability and reduced toxicity. With the three-dimensional crystal structure of efflux pumps available, further computational studies could also be useful to identify the molecular interaction of these compounds with such MDR pumps.

Current challenges for EPIs as therapeutic agents

Even though EPIs have been in laboratory experimentation since the 1990s, these are one of the futuristic prospects in our struggle against antibiotic-resistant bacteria. However, the path leading to a successful commercial EPI has a lot of roadblocks. These challenges are diverse in nature ranging from scientific and academic to administrative and economic. A major hurdle in developing and marketing an EPI is its economic worth. Major players in the pharmaceutical sector tend to stay away from this field as EPI is ultimately a new chemical entity (NCE). The drug experts are well versed with the problems associated with NCE which is trumped by the idea of modifying the currently known antibiotics that, in turn, have a well-documented pharmacological profile and clinical data from numerous patient records[102]. Academicians have looked for EPIs from both natural and synthetic compounds, however, their commercial production has not been taken under consideration at the laboratory level. The naturally derived EPIs have a complex and bulky structure making it difficult to synthesize. While synthetic molecules are easier to synthesize, these often suffer from poor solubility, toxicity and problems with cell permeability. The discovery of NCE is a demanding process in terms of capital and time as well. A considerable effort is also lost in satisfying the regulatory conditions that are extremely stringent. This, combined with average economic returns, makes the discovery of EPIs, and NCE in general, a financially infeasible venture keeping most of the pharmaceutical companies away.

A therapy using EPIs would essentially be a combination therapy. This puts another challenge of compatibility of the EPI and the antibiotic partner. The pharmacokinetics of both the partners must complement each other for a successful therapeutic

Table. List of efflux pump inhibitors (EPIs) from various sources

EPIs	Target efflux pump(s)	Bacterial strain(s)	Substrate(s)	References
Natural EPIs from plant sources				
Pheophorbide A	NorA, MexAB-OprM	*Streptococcus aureus, Pseudomonas aeruginosa*	Berberine, ciprofloxacin	50
5′-MHC	NorA	*S. aureus*	Berberine	37, 38
Carnosic acid	MsrA	*S. aureus*	Erythromycin	41
Carnosol	MsrA, TetK	*S. aureus*	Tetracycline	41
Cathinone	acrAB-TolC	*Salmonella* Typhimurium	Ciprofloxacin	51
Theobromine	acrAB-TolC	*S.* Typhimurium, *Klebsiella pneumoniae*	Ciprofloxacin, tetracycline	51
Reserpine	NorA, TetK, MepA, Bmr	*S. aureus, Bacillus subtilis, Streptococcus pneumoniae*	Norfloxacin, ciprofloxacin, tetracycline	30, 52
	ABC: Rv2936-Rv2937-Rv2938 (DrrABC) Rv0933 (PstB) Rv2686c-Rv2687c-Rv2688c RND: Rv0678, Rv1145, Rv1146, Rv2942 (mmpL7) MFS: Rv1410c (P55), Rv1877 Rv2846c SMR: Rv3065 (mmr)	*Mycobacterium* spp.	Ciprofloxacin, ofloxacin	53
4’,5’-O-dicaffeoylquinic acid	NorA	*S. aureus*	Berberine, norfloxacin	54
Curcumin	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	55
Kaempferol	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	56
N-trans-feruloyl 4’-O-methyldopamine	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	57
Silibinin	NorA	*S. aureus*	Norfloxacin	49
Genistein, Isoflavone	NorA	*S. aureus*	Berberine	38
Artesunate	AcrAB-TolC	*Escherichia coli*	Penicillin G; ampicillin, cefazolin, cefuroxime, cefoperazone	58
Orizabins	NorA	*S. aureus*	Norfloxacin, berberine	49
Resin glycosides (Orizabins IX, Murucoidins, Stoloniferin)	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	59
Citropten and furocoumarins	NorA, ErmA, ErmB	*S. aureus*	Norfloxacin, ciprofloxacin	60

Contd...

EPIs	Target efflux pump(s)	Bacterial strain (s)	Substrate(s)	References
Natural EPIs from plant sources				
Coumarins	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	61
Crysoplenol and Crysoplenetin	NorA	*S. aureus*	Berberine, norfloxacin	62
Diosmetin	MsrA, NorA	*S. aureus*	Erythromycin, norfloxacin	63
Murucoidins	NorA	*S. aureus*	Norfloxacin	59
Chrysosplenol-D	NorA	*S. aureus*	Berberine	62
Phenylpropanoid	Rv1145, Rv1146 Rv1877, Rv2846c Rv3065(mmr)	*Mycobacterium* spp.	Et-Br	64
Compound 1	NorA	*S. aureus*	Norfloxacin	65
Essential oils (Salvia species)	Tet (K)	*Staphylococcus epidermidis*	Tetracycline	66
Spectinamides	Rv1258c	*Mycobacterium* spp.	Clarithromycin, Doxycycline and Clindamycin	67
Diterpenes (ferruginol)	MsrA, TetK, NorA	*S. aureus, Mycobacterium* spp.	Tetracycline, erythromycin, norfloxacin isoniazid	68
Totarol	MsrA, TetK	*S. aureus, Mycobacterium* spp.	Erythromycin, isoniazid	69
Boeravinone B	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	70
α-Terpinene	TetK	*S. aureus*	Tetracycline	71
Biochanin A	NorA	*S. aureus*	Berberine, norfloxacin	38
Cumin seed oil, cuminaldehyde	LmrS	*S. aureus*	Et-Br	72
Epigallocatechin gallate, Epicatechin gallate	TetK	*S. aureus*	Tetracycline	39,40
Galbanic acid	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	73
Orobol	NorA	*S. aureus*	Berberine	38
Baicalein	NorA, TetK	*S. aureus, E. coli*	Ciprofloxacin, tetracycline	35,36
Tannic acid	TetK, NorA	*S. aureus*	Tetracycline, norfloxacin	74
Conessine	MexAB-OprM, AdeIJK	*Pseudomonas aeruginosa, Acinetobacter baumannii*	Cefotaxime, levofloxacin, tetracycline, novobiocin and rifampicin	75,76
Linoleic and oleic acids	MsrA	*S. aureus*	Erythromycin	77
Tiliroside, kaempferol-3-O-b-d-(6-E-p-coumaroyl)Glucopyranoside	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	78

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EPIs	Target efflux pump(s)	Bacterial strain (s)	Substrate(s)	References
Natural EPIs from plant sources				
Capsaicin (8-methyl-N-vanillyl-6 nonenamide)	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	79
Caeffeoylquinic acid	NorA	*Enterococcus faecalis, S. aureus*	Berberine	54
Piperine	NorA, MdeA, Rv1258c	*S. aureus, Mycobacterium* spp.	Norfloxacin, ciprofloxacin	33,34
Clerodane diterpene 16α-hydroxycleroda-3,13 (14)-Z-dien-15,16-olide	norA, norB, norC, mepA, mdeA	*S. aureus*	Norfloxacin, ciprofloxacin	80
Chalcone	NorA	*S. aureus*	Berberine, norfloxacin	30
Olaanolic acid, Ulvaol	NorA	*S. aureus*	Norfloxacin, oxacillin	81
Quercetin	Rv3065(mmr)	*Mycobacterium* spp.	-	53
Tetrandrine	Rv2459 (jefA), Rv3728 Rv3065(mmr)	*Mycobacterium* spp.	Isoniazid and ethambutol	53
Farnesol	-	*Mycobacterium* spp.	Et-Br	53
Synthetic EPIs (Chemically synthesized)				
4-acetyl-3-(4-fluorophenyl) -1-(p-tolyl)-5-methylpyrrole	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	82
N-trans-3,4-O dimethylcaffeoyl Tryptamine	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	83
5,7 deoxyhydnocarpin-D (5,7-DHC-D)	NorA	*S. aureus*	Berberine	31
Chalcone and derivatives	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	84
4-phenoxy-4'-dimethylaminoethoxy chalcone, (4-DAEC)	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	57
SK-20 and SK-56 (Piperine analogs)	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	33
SLUPP-225, SLUPP-417	AcrAB-TolC	*E. coli*	Novobiocin and erythromycin	85
PAβN	AdeFGH	*A. baumannii*	Trimethoprim, chloramphenicol and clindamycin	86
NMP (1-(1naphthylmethyl)-piperazine)	AdeABC, AcrAB, AcrEF	*A. baumannii, E. coli, Enterobacter aerogenes, K. pneumonia*	Levofloxacin	28
5-MPC	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	83
Verapamil	(efpA [Rv2846c], Rv1258c, jefA [Rv2459], and P55 [Rv1410c]) and (Rv1819c and pstB [Rv0933]	*M. tuberculosis*	Isoniazid	25, 26, 53
Piperazine Arylideneimidazolones	AcrAB Tol-C and AcrEF	*E. coli*	Fluoroquinolones	87

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EPIs	Target efflux pump(s)	Bacterial strain (s)	Substrate(s)	References
Synthetic EPIs (Chemically synthesized)				
Ethyl 6-amino-1 cyclopropyl-7-[4-(hydroxyimino)-3-methyl-3,4,7,8-tetrahydro-2H-thiopyrano[3,2-c] pyridin-6 (5H)-yl]-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (EDCQ)	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	83
10-(4-(-3-phenylureido)-benzylamino)-9-fluoro-3,7-dihydro-3-methyl-7-oxo-2H-[1,4] oxazino[2,3,4-ij] quinoline-6-carboxilic acid (Q6CA)	NorA, MepA	*S. aureus*	Norfloxacin, ciprofloxacin	68
Pyridoquinolines	AcrAB-TolC	*E. aerogenes*	Norfloxacin	44
2-phenyl-4-hydroxyquinoline derivativesN, N-diethyl-2-{[2-(4-propoxyphenyl) quinolin-4-yl] oxy}-ethanamine hydrochloride (PPQE)	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	46
4-(2-piperidin-1-ylethoxy)-2-(4 propoxyphenyl) quinoline (PPQ)	NorA	*S. aureus*	Norfloxacin, ciprofloxacin	46
4-(2-(piperazin-1-yl) ethoxy)-2-(4-propoxyphenyl) quinolone - PQQ4R	AcrAB-TolC	*E. coli*	Ofloxacin, tetracycline	88
(Z)-5-(2,4-dimethoxybenzylidene)-3-(2-hydroxy-3-(isopropylamino) propyl) imidazolidine-2,4-dione	AcrAB-TolC	*E. aerogenes*	Chloramphenicol, nalidixic acid and sparfloxacin	89
5-nitro-2-phenylindole, (INF 55, INF 240, INF 240, INF 271, INF 277)	NorA	*S. aureus*	Ciprofloxacin	83
[4-benzyloxy-2-(5-nitro-1H-2-yl)-phenyl]-methanol (BNPM)	NorA	*S. aureus*	Berberine, norfloxacin	83
2-phenylbenzo[b] thiophene-3 carboxaldehyde (2-PTC)	NorA	*S. aureus*	Ciprofloxacin	83
3-(3,4-dihydronapth-2-yl)-propenoic acid isobutyl amide (3-PIA)	NorA	*S. aureus*	Ciprofloxacin	83
2-((2-(4-propoxyphenyl) quinolin-4-yl) oxy) alkylamines 1-4	NorA	*S. aureus*	Ciprofloxacin	46
13-cyclopentylthio-5-OH-TC (13-CPTC), semisynthetic tetracycline (TC) analogs	TetA or TetB	*E. coli*	Tetracycline	90
Cholecalciferol and alpha-tocopherol	TetK, MsrA	*S. aureus*	Erythromycin, tetracycline	91
Phe-Arg-β-naphthylamide (MC-207, 110)	MexAB-OprM	*P. aeruginosa*	Levofloxacin	19
Biricodar, G-918	NorA	*S. aureus, E. faecalis*	FQs, Norfloxacin	49
Timcodar	-	*S. aureus, Mycobacterium* spp.	Norfloxacin, isoniazid, rifampicin	49
SILA 421	mdr-1	*Mycobacterium* spp.	-	92

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EPIs	Target efflux pump(s)	Bacterial strain (s)	Substrate(s)	References
Synthetic EPIs (Chemically synthesized)				
Phenothiazine and its derivatives (methylene blue, promethazine, chlorpromazine and thioridazine)	NorA, AcrB	*S. aureus, E. coli*	Norfloxacin, FQs	49
	-	*Burkholderia pseudomallei*	Erythromycin, levofloxacin and azithromycin	
Chlorpromazine	AcrB	*S. enterica*	Et-Br	49
phenyl-1,4-benzothiazine derivatives	NorA	*S. aureus*	Ciprofloxacin	93
Pyridoquinolines	AcrAB-ToIC	*K. pneumonia, E. aerogenes*	Tetracycline, norfloxacin, chloramphenicol	44
2-(4-Propoxy-phenyl) quinolone derivatives	NorA	*S. aureus*	Ciprofloxacin	46
Valinomycin	Rv1410c (P55)	*Mycobacterium* spp.	Isoniazid	57
Pyridopyrimidine analogues (D13-9001, D2)	AcrB and MexB	*E. coli, P. aeruginosa*	FQs	49
Pyranopyridine derivatives (MBX2319)	AcrAB	*E. coli*	Ciprofloxacin	29
(E)-N-(3,4-difluorophenyl)-2-(2-(3-(methylthio) phenylimino)-4-oxothiazolidin-5-yl	AbeM	*A. baumannii*	Norfloxacin, ciprofloxacin	20
DHA7, DHA 27	AcrB	*E. coli*	FQs	94
Riparin-B	NorA	*S. aureus*	Ciprofloxacin, norfloxacin	95
Nerol, Dimethyl octanol and Estragole (monoterpenes)	NorA	*S. aureus*	Norfloxacin	96
PA EPA amides	NorA	*S. aureus*	Norfloxacin	97
6-(aryl) alkoxypyridine-3-boronic acids, 6-(3-Phenylpropoxy) pyridine-3-boronic acid 3i and 6-(4-phenylbutoxy) pyridine-3-boronic acid 3j	NorA	*S. aureus*	Ciprofloxacin	98
Ginsenoside 20(S)-Rh2 (Rh2)	NorA	*S. aureus*	Ciprofloxacin	99
Pimozide (neuroleptic drug)	AcrAB-TolC	*E. coli*	Et-Br	100
Sertraline	AcrAB, AcrEF, MdtEF and MexAB	*E. coli*	Levofloxacin, tetracycline	45
EPIs from microbial sources				
EA-371α and EA-371δ	MexAB-OprM	*P. aeruginosa*	Levofloxacin	101

PA, piperic acid; EPA, 4-ethylpiperic acid; DHA7, dihydroartemisinine 7; PaβN, Phenylalanine-arginine β-naphthylamide; 5′-MHC, 5'-methoxyhydnocarpin

combination[102]. These considerations are often neglected in laboratory experiments, but these assume extreme importance from the clinical point of view. For example, the combination of verapamil, a Ca^{++} channel blocker, with clarithromycin, a macrolide antibiotic, has been observed to be fatal, with the US FDA issuing a strict warning[103]. The target of clarithromycin is a cytochrome that is responsible for metabolism of verapamil. The combined use of both the drugs could lead to accumulation of verapamil at extremely toxic levels leading to kidney failures, hypotension and death[103].

A major challenge for EPIs as therapeutic agents itself lies with their targets. Efflux pumps are one of the mechanisms but not always the only mechanism of antibiotic resistance. In bacteria such as *A. baumannii* and *P. aeruginosa*, the fluoroquinolone resistance is often mediated by the efflux pumps as well as point mutations in the gyrase-coding genes[104].

The problem is compounded by co-expression of multiple pumps and substrate redundancy. This makes the EPI-antibiotic combinatorial therapy case-specific and casts doubts over the success at the community level.

While EPIs usually show promise with an antibiotic against the efflux pump, it is often seen that the same EPI does not potentiate the activity of other substrates of the same efflux pump. PAβN is effective at potentiating only a certain set of antibiotics while it does not really potentiate other substrate antibiotics of the pump MexAB[19]. Like PAβN, many EPIs are substrates of the pumps and act at a particular substrate-binding site. An indirect implication of this observation is that a high concentration of EPI would be required to ensure that these competitively prohibit the interaction of substrate antibiotics with the pump. Unfortunately, the fare well with antibiotics that are also the substrate of the pump but have a different substrate-binding site. This greatly narrows the spectrum of an EPI, making it highly specific for only a limited number of substrates. Although it is difficult to discover an NCE that inhibits the efflux of antibiotic from a pump, it is extremely hard to find an EPI that would inhibit multiple pumps across multiple bacterial species. Although some molecules have a common mechanism of inhibition, these have been found to inhibit animal efflux pumps as well, resulting in toxicity and unfavourable pharmacological profile[102].

Other challenges that plague the success of EPIs stem from the lack of pre-clinical and clinical data. There is a limited amount of information on model organisms and patient data to support the activity of EPIs. More work at the pre-clinical and clinical level is required to take the EPI research to the next level[102]. No to low frequency of mutant generation is one of the advantages of using EPIs. However, random mutagenesis using PCR has resulted in efflux pump variants that retain their activity but are resistant to the action of EPI[32]. Although it seems a rare possibility, it cannot be denied that under an immense selection pressure, bacteria may develop such modifications that ultimately save them from the EPI-antibiotic combination therapy.

Future perspectives

Although the use of EPIs as therapeutic agents faces a lot of challenges, that should, in no way, undermine the importance and advantage they offer. In times where the antibacterial pipeline has almost dried out, EPIs provide a ray of hope by rejuvenating the activity of already available antibiotics. The use of EPIs obviates the discovery of new antibiotics, a strategy that saves a lot of time, effort and capital associated with discovery of a novel antibiotic. It allows the clinicians to exploit the already well-established pharmacological properties of known antibiotics. A very important implication of EPIs as therapeutic agents is the ability to reverse antibiotic resistance. It assumes great importance when we consider the fact that the current economic conditions also favour the large-scale production of already optimized and stockpiled antibiotics. Another striking advantage of using EPIs is the extremely low frequency of generation of resistant mutants. The combination of antibiotic and EPI is, therefore, effective in not only tackling the already resistant bacteria but also providing respite from the future problems of development of resistance.

Evaluating the potential of EPIs, it appears that although the use of EPIs is an attractive strategy, it is far from realization yet. There are many gaps that need to be plugged and a lot of distance to be covered. The technical downsides and limitations of the EPIs need urgent attention. More research is required to highlight the scientific and economic merit of EPIs. This would ultimately help in attracting the interest of pharmaceutical industries and more capital. To sum up, there is a considerable amount of effort currently underway at the bench level; however, it will take more consideration and effort before the EPIs can finally make it to the bedside.

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