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Abdallah Mahamoud[1], Jacqueline Chevalier[1], Sandrine Alibert-Franco[1], Winfried V. Kern[2] and Jean-Marie Pages[1]*

[1]UMR-MD-1, Facultes de Medecine et de Pharmacie, Universite de la Mediterranee, 27 Boulevard Jean Moulin, F-13385 Marseille Cedex 05, France; [2]Center for Infectious Diseases and Travel Medicine, University Hospital, D-79106 Freiburg, Germany

After several decades of continuously successful antibiotic therapy against bacterial infections, we are now facing a worrying prospect: the accelerated evolution of antibiotic resistance to important human pathogens and the scarcity of new anti-infective drug families under development. Efflux is a general mechanism responsible for bacterial resistance to antibiotics. This active drug transport is involved in low intrinsic susceptibility, cross-resistance to chemically unrelated classes of molecules, and selection/acquisition of additional mechanisms of resistance. Thus, inhibition of bacterial efflux mechanisms appears to be a promising target in order to (i) increase the intracellular concentration of antibiotics that are expelled by efflux pumps, (ii) restore the drug susceptibility of resistant clinical strains, and (iii) reduce the capability for acquired additional resistance. Structurally unrelated classes of efflux pump inhibitors (EPIs) have been described and tested in the last decade, including some analogues of antibiotic substrates and new chemical molecules. Among the current collection of EPIs, only a few compounds have been studied taking into account the structure–activity relationships and the spectrum of activity in terms of antibiotics, pumps and bacteria. While large efforts have characterized an increasing number of bacterial efflux pumps and generated several potentially active EPIs, they have not elucidated the molecular basis of efflux transport and inhibition. Recent studies of pump–substrate complexes, the 3D resolution of the efflux pumps, the synthesis of novel compounds and molecular dynamic studies may generate new clues to decipher and select novel targets inside the efflux mechanisms and, finally, may result in a clinically useful molecule.

Keywords: antibiotic resistance, drug efflux pumps, efflux pump inhibitors

Efflux pumps and antibiotic resistance

Bacterial resistance to antibiotics such as β-lactams, aminoglycosides and quinolones can be summarized as different, interacting steps based on the discovery of novel molecules belonging to various antibiotic classes, their clinical use, and the characterization of emerging resistance mechanisms. A consequence of these tight relationships and increasing antibiotic resistance is the urgent requirement to develop novel molecules that are insensitive to resistance processes in order to combat resistant pathogens. The accelerating increase of bacterial resistance and resultant problematic therapy are directly responsible for the current increase in morbidity and mortality associated with bacterial infections.

Among the various mechanisms involved in bacterial resistance, the balance of membrane permeability which controls the traffic (in and out) of various molecules, plays a key role in the influx and efflux of antibiotics, thereby limiting their intracellular concentration. Multidrug resistance efflux pumps have now been described in both Gram-positive and Gram-negative bacterial pathogens. The poly-specificity of efflux transporters confers a 'general resistance mechanism' that can reinforce the effect, and/or favour the acquisition, of other mechanisms of antibiotic resistance such as mutations of the antibiotic targets or modification of the drugs.[1–5] Gram-negative bacteria become, in general, more readily resistant to antibiotics owing to the sophisticated architecture of their cell envelope, including the outer and inner membranes which delineate the periplasmic space. A toxic compound can be picked up in the periplasm and expelled directly to the external medium, strongly reducing the number of molecules reaching their cytoplasmic targets.[1–7] Conjointly to these, the external barrier to diffusion, the outer

*Corresponding author. Tel.: þ33-4-91-32-45-87; Fax: þ33-4-91-32-46-06; E-mail: Jean-Marie.PAGES@medecine.univ-mrs.fr

membrane, acts as an additional protective step which strongly limits the passive penetration of hydrophilic, charged and hydrophobic molecules.[8,9] Moreover, because this envelope architecture is responsible for additional levels of resistance to some antibiotic classes (e.g. b-lactams, fluoroquinolones), Gram-negative bacteria are a major cause of antibiotic-resistant infections.

The complex envelope of Gram-negative bacteria contains a variety of protein channels involved in the transport (influx or efflux) of a large variety of nutrients (sugars, amino acids, salts, metals, etc) or noxious compounds (metabolites, drugs, biocides, detergents, etc.). Among these transporters are distinct energy-dependent efflux pumps that recognize toxic agents such as antibiotics and extrude (pump out) the agent from the periplasm/cytoplasm to the exterior (environment) of the cell, thereby reducing the intracellular accumulation of the agent. Overexpression of one or more of these efflux pumps prevents the intracellular accumulation of the agent to thresholds necessary for its inhibitory activity.[1–6] This efflux pump overproduction is generally accompanied by an increase in resistance to two or more structurally unrelated antibiotics [multidrug-resistance (MDR)] and significantly contributes to the emergence and spread of MDR pathogens. The mechanisms of influx and efflux cooperate in the control of intracellular concentration by various efficient regulation cascades.

Phylogenetically, bacterial antibiotic efflux pumps belong to five superfamilies (see http://www.biology.ucsd.edu/~msaier/transport/ for classification, and reviews[2,10,11]), namely: (i) ABC (ATP-binding cassette), which are primary active transporters energized by ATP hydrolysis; (ii) SMR [small multidrug resistance subfamily of the DMT (drug/metabolite transporters) superfamily]; (iii) MATE [multi-antimicrobial extrusion subfamily of the MOP (multidrug/oligosaccharidyl-lipid/polysaccharide flippases) superfamily]; (iv) MFS (major facilitator superfamily); and (v) RND (resistance/nodulation/division superfamily), which are all secondary active transporters driven by ion gradients. The MFS and RND pumps are the most abundant: the MFS pumps are found in both Gram-positive and Gram-negative bacteria, and are characterized by a relative narrow spectrum, recognizing usually one or sometimes a few antibiotic classes; the RND pumps are found exclusively in Gram-negative bacteria and display an extremely wide spectrum of substrates (poly-selectivity), including not only several classes of antibiotics, but also antiseptic compounds, dyes, or detergents.[1–7]

These pumps have been discussed in detail (regulation and expression, topology, presence in bacterial species, main substrates, etc.) in recent reviews.[1–7,12–16] In this article we will focus on efflux pump inhibitors (EPIs) that are active against resistant Gram-negative bacteria.

Circumventing the efflux mechanism: an emerging strategy to face an emerging problem

It comes as no surprise that efflux mechanisms responsible for the decreased effectiveness of common antibiotics also account for the resistance to new, recently described antimicrobial agents such as a peptide deformylase inhibitor,[17] plectasin[18] and platensimycin.[19] This strongly supports the need for research and development into compounds that are able to circumvent or block efflux pumps and to restore/preserve antibacterial potency of older as well as newer antibiotics.[20–25] Moreover, it has been recently demonstrated that the expression of AcrAB-TolC pump is an important prerequisite for the selection of ciprofloxacin-resistant mutants.[26] Similar observations have been reported concerning the role of the CmeABC efflux pump in the emergence of fluoroquinolone-resistant Campylobacter strains.[27]

Response to drug efflux mechanisms: a present challenge

In order to address the problem of efflux pumps and their consequences on decreasing the intracellular active concentration of antibiotics, it is necessary to search for and develop new molecules to circumvent efflux activity. Different strategies to reach this objective are:

(1) By-passing efflux activity: improving the molecular design of old antibiotics to reduce their efflux. The profile of antibiotics will be changed:
   - † by modifying the structure of a molecule in order to decrease its affinity for the affinity sites located inside the efflux pump;
   - † by introducing new side chains in order to block/impair the transport.

(2) Direct action on the permeability of the bacterial cell envelope: decreasing the efficacy of the membrane barrier. The permeability of the cell envelope will be modified by:
   - † a direct modification, via a detergent-like effect, ensuring an improved uptake that yields increased intracellular concentration of the antibiotic;
   - † a channel-blocker that induces a 'traffic jam' in the outer membrane channel (TolC) restoring a high intracellular drug concentration.

(3) Blocking the efflux capacity of bacterial cell: alteration of pump function. The reversal of resistance will be obtained by using:
   - † competitive inhibition: competition between an agent that uses the same substrate (ligand) site inside the pump and the antibiotic(s);
   - † non-competitive inhibition: poly-selective binding to pump components producing steric hindrance within the cavities of the pump;
   - † energy wasting, direct/specific via a antiporter site or indirect/general via a collapse of energy driven mechanisms of the bacterial cell envelope.

Concerning strategy (1), no novel drug has been designed according to the recent criteria obtained from the determination of pump structures. However, the recent advances in bacterial genomics, enabling resolution of the 3D structure of efflux pumps, and the combinatorial chemistry will provide new means, tools and concepts for the improvement of existing antibiotics.

Decreasing the efficacy of the membrane barrier: permeabilizing compounds

Concerning permeabilizing agents, one molecule has presented some attractive properties, the polymyxin B nonapeptide.[28] This molecule and neopeptide antibiotics, which exhibit a structure close to the polymyxin class with a reduced direct bactericidal activity, are able to destabilize the membrane allowing the

enhanced penetration of antibiotics into the bacterium.[28,29] The cyclic moiety of the molecule binds to the Gram-negative lipopolysaccharide and permeabilizes the outer membrane to hydrophobic antibiotics as well as to other bactericidal agents.[29] The use of these molecules is limited by the same adverse effects produced by polymyxin-type antibiotics such as acute nephrotoxicity, although some of the most recent polymyxins are less toxic.[29] However, more investigations are necessary to elucidate the conditions necessary to restore the activity of conventional antibiotics against multidrug-resistant (MDR) bacteria. Because various detergents, such as SDS and Triton-X, which affect the integrity of the cell envelope, are also substrates of efflux mechanisms due to their lipophilic and amphipathic properties,[2] the use of such agents may be limited unless they can be modified in a manner that renders them non-substrates.

What about specifically blocking the TolC—or other similar family channel? Although this possibility seems to be attractive, especially with regard to their dual role in the secretion of virulence factors,[30] this approach has not been really investigated to the authors' knowledge. However, study of this interesting approach will be facilitated by the recent data concerning the functional organization and the structure of the AcrAB-TolC efflux pump.[31–33]

The efflux pump energy: targeting the driving force of the mechanism

Compounds that seriously affect the energy level of the bacterial membrane such as carbonyl cyanide m-chlorophenylhydrazone (CCCP), are used in the laboratory to abolish totally the efflux of various molecules.[23,34,35] These compounds reduce the viability of the bacterium and cause cell death via the dissipation of the proton-motive force of the membrane. Consequently, there is always the question of whether it is their effect on the efflux pump that is the cause of an increase in the penetration of the antibiotic, or whether it is due to the alteration of the cell envelope itself that results in the death of the bacterium. In addition, some of them, like CCCP, are recognized as highly noxious and cytotoxic and are also substrates of bacterial efflux pumps. Today, no molecule belonging to the energy-blocker family has been developed for clinical use or has been patented.

What about general inhibitors of efflux pumps such as reserpine and verapamil? These molecules were initially documented as inhibitors of vesicular monoamine transporters and blockers of transmembrane calcium entry (or calcium ion antagonists), respectively. Verapamil is an inhibitor of MDR pumps of cancer cells and parasites and also improves the activity of tobramycin.[2] Reserpine inhibits the activity of Bmr and NorA, two Gram-positive efflux pumps.[2] They alter the generation of the membrane proton-motive force required for the function of MDR efflux pumps. Although these molecules are able to inhibit the ABC transporters involved in the extrusion of antibiotics (i.e. tetracycline), the concentrations necessary to block bacterial efflux are neurotoxic.[2] Even though reserpine has been used as an anti-hypertensive drug, its concentration for this purpose is far lower than that employed for the inhibition of efflux. Verapamil and reserpine are routinely used to evaluate the activity of efflux pumps in Gram-positive bacteria.[2]

Chemically synthesized derivatives and natural products such as 5-methoxyhydnocarpin or berberine have activities against efflux pumps.[20,36] Problems with their synthesis, purification, stability and solubility, in addition to their potential toxicity, have reduced interest in further development of these promising compounds as efflux pump inhibitors.

Alteration of pump function: the flux inhibitors

Peptidomimetics

From the study of efflux mechanisms acting on Gram-negative antibiotic-resistant strains of Pseudomonas aeruginosa, Microcide and Daiichi Pharmaceuticals have produced a large family of peptidomimetics that exhibit EPI properties (Table 1). Among this family, the first identified EPI from a screening based on the ability to restore levofloxacin susceptibility in various P. aeruginosa clinical strains, was MC-207 110 or phenylalanine arginyl b-naphthylamide (PAbN). In addition to

Table 1. Known inhibitors of Gram-negative efflux pumps

Type	Substrates	Bacteria	SAR	Reference
Phenothiazine	tetracyclines	Escherichia coli	−	67
Phenylpiperidine	norfloxacin, tetracycline, ethidium bromide	E. coli	−	59
Tetracycline analogue	tetracyclines	E. coli	−/+	64,65
Aminoglycoside analogue	tetracyclines, gentamicin	Haemophilus influenzae	−	25
Fluoroquinolone analogue	fluoroquinolones, macrolides	E. coli, Pseudomonas aeruginosa	−	25
Quinoline derivative	chloramphenicol, norfloxacin, tetracycline	Enterobacter aerogenes, Klebsiella pneumoniae	+	52
Peptidomimetic	quinolones, chloramphenicol, macrolides, carbenicillin, tetracycline	P. aeruginosa, E. aerogenes, E. coli, K. pneumoniae, Acinetobacter baumannii, Campylobacter jejuni	+	24
Pyridopyrimidine	fluoroquinolones, b-lactams	P. aeruginosa	+	46–50
Arylpiperidine	linezolid	E. coli	−/+	58
Arylpiperazine	fluoroquinolones, tetracyclines, macrolides, linezolid, chloramphenicol	E. coli, Citrobacter freundii, K. pneumoniae, E. aerogenes, A. baumannii	−/+	60–63

SAR, studies on structure–activity relationships; +, available; −/+, partial data; −, no data.

its effect on levofloxacin activity, PAbN is also reported to restore the activity of various other antibiotic classes, including chloramphenicol and macrolides.[24,37–39] The ability of this agent to restore antibiotic susceptibility of resistant bacteria is attributed to its inhibiting the efflux of one or more antibiotics,[24] and therefore the molecule can be considered to exhibit a broad spectrum of efflux pump inhibition.

The analyses of the inhibitory mechanism have demonstrated that this EPI is a substrate of efflux pumps and may act as a competitive inhibitor.[24] These studies, in addition to the recent work describing the co-crystallization of AcrB with various substrates,[40,41] support the idea that MC-207 110 may recognize and bind to the same affinity pocket used by the potentiated antibiotics (e.g. levofloxacin) located inside the AcrB cavity or inside the MexB cavity in P. aeruginosa. Alternatively, due to a close location of binding site, the EPI binding may also generate steric hindrance, impairing the antibiotic binding at its affinity site. In order to clarify these possibilities, affinity constants and kinetic parameters should be determined (see the concluding section 'A future for combating efflux pumps'). The pharmaceutical companies Essential Therapeutics and Daiichi Pharmaceutical have produced MC-207 110 derivatives exhibiting structural modifications in order to improve the biological stability of EPIs and to enhance the therapeutic and pharmacodynamic profiles (Table 1). Some of them[37,42,43] result from the substitution of an amino acid unit (Orn in place of Arg), or use D-amino acid residues (D-Orn in place to L-Orn). Additional structural modifications have provided derivatives for in vivo evaluations. In parallel studies, structure–activity relationships have examined the role of the peptidic backbone present in this inhibitor family.[42–46] In addition to these MC-207 110 derivative compounds, pyridopyrimidines have been developed as EPIs in P. aeruginosa.[47–51]

A major problem with members of this EPI family is their toxic properties, which prevents their clinical application.[23–25] However, these products are widely used to experimentally determine and evaluate the efflux mechanisms of bacterial pathogens. In addition, they are used to measure the efflux activity and determine the level of inhibitor-sensitive efflux for specific antibiotics in various bacteria.[24,39]

Quinoline derivatives

This novel class of compounds was discovered by using several screening procedures with Enterobacter aerogenes strains.[52,53] These compounds have been assayed for their activity against various MDR clinical strains overexpressing efflux pumps that expel different antibiotics (e.g. chloramphenicol, norfloxacin). Active quinoline derivatives have then been evaluated for their ability to restore the activity of various antibiotic families (e.g. quinolone, phenicol, cycline).[52–57] Data from many sources confirm the potential of these EPI compounds, and several quinoline derivatives are now considered as broad-spectrum EPIs for rendering antibiotic-resistant E. aerogenes and Klebsiella pneumoniae susceptible to chloramphenicol, tetracycline and norfloxacin (Table 1).[56] Moreover, the direct action of this family of molecules on the drug extrusion mechanism has been clearly demonstrated by measuring the intracellular concentrations of antibiotics (norfloxacin, chloramphenicol) after their addition to bacterial cultures; their ability to increase the accumulation of the antibiotic has been compared to that resulting from the addition of CCCP or PAbN to the culture.[52–57]

An interesting point to note is the relatively poor efficacy of these products against the MexAB-OprM efflux pump of P. aeruginosa (J. Chevalier, A. Mahamoud and J.-M. Pagès, unpublished results) if compared with the substantial activity of PAbN to reverse drug resistance in this bacterium. This observation could be attributable to differences in the sequences of AcrB and MexB, which may induce some change in the functional organization of the efflux pump; these modifications may affect amino acid residues that are directly involved in the substrate affinity sites, e.g. in the transport of EPIs or antibiotic molecules in both E. aerogenes and P. aeruginosa. In addition, the original screening protocols are different, at least for the selected bacterial efflux target and for the antibiotic used as substrate (levofloxacin versus chloramphenicol), for the two EPI families (peptidomimetics or quinoline derivatives).[37,38,52]

The analyses of structure–activity relationships have indicated that the alkyl side-chain linked to the heterocyclic moiety of alkylaminoquinolines plays a key role in EPI activity.[52] Alkoxy- and thioalkoxy-quinolines having piperidinoethyl chains restore susceptibility to chloramphenicol, with the degree of susceptibility increased depending upon the type of derivative—less so with oxo-derivatives than with amino substitutions in thioalkyl molecules.[52] In addition, the connecting heteroatom and the position of substituted groups on the ring seem also to be of importance. In this inhibitor class, additional studies are necessary to define the role of pharmacophoric groups and their reactivity with the affinity pockets reported in AcrB.[31,32,41] Alkylamino-, alkoxy-, thioalkoxy-, chloro-quinoline derivatives present two advantages: their similarity with the quinolone family, which greatly argues for an efficient pharmacokinetic profile and a negligible intrinsic activity, and no additional side-effect (permeabilization or alteration) on the membrane.[52] However, toxicity assays and pharmacodynamic studies are still needed to determine the therapeutic potency of these compounds.

Arylpiperidines and arylpiperazines

Several compounds belonging to these two families have been tested as potential EPIs (Table 1). Among arylpiperidines, some derivatives (dihalogens) are able to restore linezolid susceptibility and linezolid accumulation in Escherichia coli.[58] In addition, phenylpiperidine selective serotonin re-uptake inhibitors inhibited the function of two unique Staphylococcus aureus multidrug efflux pumps and also partially affected the activity of the AcrAB-TolC pump in E. coli.[59] Concerning arylpiperazine, screening of a limited library of low-molecular-weight N-heterocyclic compounds in E. coli led to the discovery of several arylpiperazines with a potency to reverse multidrug resistance in cells overexpressing RND-type efflux pumps.[60] Elongation of the spacer between the benzene ring and the piperazine ring as well as halogenic substitutions at the benzene ring enhanced the potency of the compounds. Despite a significant sensitizing effect, some of these compounds were strikingly poor in their efficiency to increase the intracellular concentration of efflux pump substrates, suggesting alternative or additional mechanisms of action. A few anthrylpiperazines and naphthylpiperazines, notably N-methylpyrrolidone (NMP), were among the most potent unsubstituted arylpiperazines, with a minimal effective concentration and a dose-dependent ability to increase the

intracellular concentration of such diverse substrates as fluoroquinolones, chloramphenicol, fluorescent dyes and linezolid. NMP was tested in several bacterial species including clinical isolates. Interestingly, the compound was also effective in Acinetobacter baumannii, in different Enterobacteriaceae except Serratia, but not in P. aeruginosa.[61–63] Due to the serotonin agonist properties of arylpiperazines, these compounds are likely to be too toxic for use in man and animals. The mechanism of action of NMP and related compounds has not been elucidated. Unlike PAbN, the (low) intrinsic antibacterial activity of NMP and its cellular accumulation are not enhanced in cells with inactivated efflux pumps.

Other efflux pump inhibitors

Various antibiotic analogues have been developed in order to circumvent the efflux pumps and restore the antibiotic activity of efflux-mediated resistant strains. Among them are tetracycline analogues which were first described by S. B. Levy's team in 1993. Initially focused on S. aureus and tetracyclines, these molecules have been tested with other antibiotics and other bacterial pathogens.[64–66] The problem with this class of modified antibiotics is its high similarity of structure to the true antibiotic and its residual activity on bacterial targets; an adverse consequence of these properties is the reinforcement of the selection of resistance mechanisms directed against the antibiotic.

In addition to the products described, several products have been patented,[25] some of them exhibiting interesting capabilities. However, their exact properties, activity, solubility, purification, stability, and toxicity remain to be clearly defined.[67,68]

A future for combating efflux pumps

It is now important to tackle a serious gap in our knowledge of efflux mechanisms. Although we have some information regarding the structure and activity of AcrAB-TolC or MexAB-OprM pumps, the archetype of drug transporters, and about some inhibitors, we know very little about the mechanical and dynamic aspects of their function. The resolution of the structure of co-crystals of AcrB with various substrates[40,41] and the recently proposed mechanisms including the 'peristaltic pump mechanism' and the 'functionally rotating ordered multidrug binding change mechanism'[27,31] are very promising but not totally satisfactory for the definition of suitable physicochemical characteristics for a future efficient inhibitor. To date, the activity of putative EPIs is deduced from various measures using the susceptibilities towards different antibiotics in the absence and presence of the agent. Similarly, the degree of inhibition of antibiotic efflux is determined by the variation of intracellular concentrations induced by the addition of tested compounds and compared to the level obtained in the presence of a poison that collapses the membrane energy necessary for the drug expulsion. To quantify the effect of EPIs on an efflux pump, more effective assays are needed for determination of kinetic parameters and their relationships to the structure of the component of the efflux pump that is affected.[52] The definition of these parameters is necessary for intelligent design of new EPIs and their eventual use for the therapy of MDR bacterial infections. In addition, these data are important in order to choose between the development of a 'general inhibitor' targeting the transporter that expels various antibiotics in one bacterial species or a 'selective inhibitor' blocking the pumping out of one antibiotic family in various bacteria—or, an intermediate solution.

To date, no efflux pump inhibitor has been licensed for use in the treatment of bacterial infections in human or veterinary medicine, and it is clear that this gap in our antimicrobial armamentarium must stimulate research that leads to the development of new EPI molecules.[23,24] The major benefits derived from developing efficient efflux pump inhibitors will be the ability to re-use various antibiotics affected by the efflux pumps as well as the control of the emergence and the dissemination of MDR efflux strains. Until now, the majority of the known inhibitors have been obtained from screening libraries of synthetic compounds, from purification of natural compounds or 'naive' chemical modification of existing molecules. With the recent resolution of the 3D structure of drug transporters, the co-crystallization of AcrB with some substrates, and the analyses of the biological effects of several mutations in the pump, we are able to begin rational design of functional targets located inside the bacterial pumps. Consequently, the synthesis of efficient molecules with strong EPI capacity will be more readily achieved. In addition, the definition of binding sites located inside the pump will be used to improve the antibiotic profile in order to decrease the affinity for pump affinity sites and residues involved in the drug transport, e.g. by introducing groups favouring steric hindrance. This may support the rationale for a new generation of antibiotics that are 'resistant or less susceptible to efflux'.

With this new generation of designed antibacterial compounds that are able to restore the antibiotic susceptibility by selectively blocking membrane transport, novel uses of transport inhibitors will emerge in order to (i) compete with the diffusion of quorum sensing molecules, or (ii) block the secretion of virulence factors.

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None to declare.

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