Strategies for bypassing the membrane barrier in multidrug resistant Gram-negative bacteria

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In Gram-negative bacteria, the envelope is a sophisticated barrier protecting the cell against exter-nal toxic compounds. Membrane transporters, e.g., porins or efflux pumps, are main filters regulat-ing the internal accumulation of various hydrophilic molecules.
Regarding bacterial susceptibility towards antibacterial agents, membrane permeability is part of the early bacterial defense. The bacterium manages the translocation process, influx and efflux, to control the intracellular concentration of various molecules. Antibiotics and biocides are substrates of these mechanisms and the continuing emergence of multidrug resistant isolates is a growing worldwide health concern. Different strategies could be proposed to bypass the bacterial membrane barrier, comprising influx and efflux mechanisms, in order to restore the activity of antibiotics

Keywords: Antibiotic
Bacterial efflux pump Chemosensitizer Drug transporter Efflux pump inhibitor
Gram-negative bacteria Lipopolysaccharide MDR bacteria Membrane
permeability Natural compound Porin
Selectivity

1. Introduction

After several decades of continued success of antibiotic therapy against bacterial infections, we are now facing a worrying prospect: the accelerated evolution of antibiotic resistance of important human pathogens [1–5]. This bacterial adaptation to antibiotic use is directly involved in the current increase of morbidity and mortality caused by infection diseases. Gram-negative bacteria that correspond to a prominent part of drug resistant pathogens, display a complex envelope with an outer (OM) and an inner (IM) membrane delimiting a periplasmic space [6]. This cellular organization results in the presence of various protein channels involved in the transport, uptake or efflux, of a large variety of compounds, nutrients or toxic molecules (sugars, drugs, small peptides, chemicals) (Fig. 1). Among these various channels, porins and efflux transporters contribute to the balance of intracellular drug accumulation and are involved in the bacterial antibiotic susceptibility [7,8].

Hydrophobic compounds, such as aminoglycosides, macrolides, rifamycins and other chemicals, can permeate through the OM bilayer by the self-promoting pathway [6]. Regarding hydrophilic molecules, such as b-lactams and fluoroquinolones, the core region of lipopolysaccharide (LPS) impairs their diffusion and porins are the major way to enter the cell [6,8]. Consequently, LPS alterations can provide an efficient protection against the first class whereas porin modifications can protect bacteria against the second ones. The permeability of porins to b-lactam antibiotics has been demonstrated by various means and several porin mutants have been described in resistant clinical isolates [8].

The over-expression of efflux pumps is involved in the Multi-Drug Resistance (MDR) phenotype associated with other more

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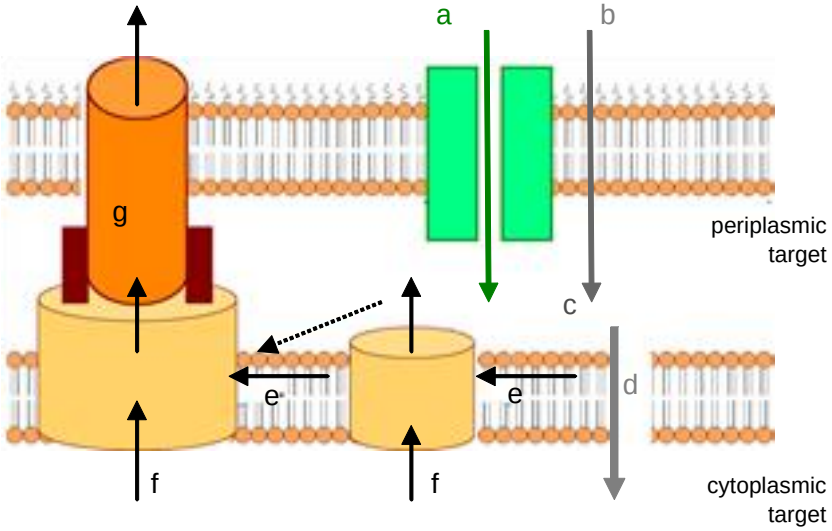


Fig. 1. Steps of antibiotic transport through the membranes of Gram-negative bacteria. To be active against bacterium, an antibiotic needs a critical concentration threshold to inhibit the corresponding target. Using the concentration gradient, the influx is controlled by several elements: the antibiotic can permeate through the OM via porins (a, for hydrophilic molecules) or through the OM (b, for hydrophobic drugs) [6]. (c) Represents the diffusion through the periplasmic space in which enzymatic inactivation may occur [26,27]. Finally, the drug will pass the cytoplasmic/IM (d). Regarding the efflux, the drug may be recognized and transported by active pump (e) or (f). The pump (e.g., AcrB) has to recruit the OM pore TolC (g), in addition it may conjointly acts with other pumps (e.g., MFS family). The success of a drug is given by the kinetics of all individual steps [12,110].

specific resistance mechanisms including target mutation and enzymatic modification of the antibiotic [9,10]. In addition to efflux pump over-expression, a severe down regulation of porin synthesis have been reported in various enterobacterial resistant isolates including Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae, etc [8]. In these strains, a complex regulation cascade is involved in the control of membrane transporters, porins and efflux pumps.

This regulation cascade involved the mar and ram genes that organize a tight and coordinated control of the expression of porins and efflux pumps in E. aerogenes, K. pneumoniae and Salmonella enteritidis [10]. Various chemicals, antibiotics, biocides, and other molecules can trigger this regulation cascade [9,10]. The main clinical concern of efflux pump expression is related to their broad polyselectivity and their ability to expel a large collection of molecules [11–14]. In Gram-negative bacteria, the archetype of drug efflux pump is the AcrAB-TolC/MexAB-OprM [15–20]. Firstly characterized in E. coli and Pseudomonas aeruginosa (Fig. 1), AcrAB-TolC/MexAB-OprM efflux pump is the main multitransporter system reported in enterobacterial clinical isolates [7,11–14]. AcrAB-TolC homologous efflux complexes have also been reported in many other pathogens [11–14]. The poly-specificity of transporters confers a "general resistance mechanism" contributing to the first line of bacterial defense that can favor the acquisition of other resistance mechanisms such as target mutations or drug modifications [10,11]. It has been demonstrated that the expression of Salmonella and Campylobacter efflux pump is an important prerequisite for the selection of fluoroquinolone resistant strains exhibiting target mutations [10,21]. The mechanics of efflux pump have been investigated and dynamic-functional models have been recently proposed [12,20,22–24]. In addition, recent data indicate that some drug transporters, e.g., MdfA and AcrAB-TolC, can cooperate or can act sequentially to efficiently expel antibiotics outside the bacterium [25].

These two transport mechanisms, influx (porin and self-promoting pathway through LPS) and efflux (efflux pumps), that can be genetically regulated by a same genetic cascade involving global regulators and specific local regulators, can cooperate to strongly decrease the intracellular concentration of antibacterial agents. These processes conjointly contribute to the membrane barrier, or mechanical barrier, that constitutes the first line of bacterial defense against antibacterial agents comprising antibiotics, biocides (disinfectants, antiseptics, preservatives), bacteriocins, detergents, surfactants, defensins, bile salts and also dyes and other chemicals.

Consequently, in order to bypass the membrane barrier existing in resistant isolates, we need to develop various strategies to increase the diffusion of antibiotics through bacterial membranes (target the influx), or/and, circumvent the pump mechanism to preserve a high intracellular concentration of antibiotics (target the efflux).

2. Increasing the antibiotic influx

During last decade, in order to restore the efficacy of b-lactams or other antibiotics in multidrug resistant strains, several ways have been investigated. In addition to explore new compounds able to inhibit cephalosporinase and other enzymatic activities [26,28], an alternate possibility is to facilitate the diffusion of antibiotics through the bacterial envelope in order to increase their intracellular concentration (see Fig. 1, arrows a and b). To bypass the barrier due to porin alterations (decreased expression or altered channel) and LPS modifications, chemical facilitators and chemosensitizers, have been proposed such as detergents, surfactants, chaotropic agents, polymyxines, antimicrobial peptides [29,30]. Some of them, e.g., polycationic cyclic lipopeptides and cationic antimicrobial peptides have been assayed in combination with usual antibiotics to combat resistant clinical strains [for recent reviews see 31–33]. It is important to note that only very few results have been published regarding the molecular basis of this kind of combination, the synergy efficacy, the concentration ratio, the kinetics, and the combination parameters.

2.1. Polymyxins as chemosensitizers

Membranotropic compounds such as polymyxin B and polymyxin E (colistin) perturb the integrity of the bacterial membranes and can increase the enterobacterial susceptibility to various compounds [33]. In addition, they can act as opsonins to enhance

destruction by phagocytic cells. These molecules are pentacationic cyclic lipodecapeptides that present bactericidal activity by acting on the outer membrane of Gram-negative bacteria [33,34]. Their action is limited to Gram-negative bacteria and polymyxin used was almost halted between 1970 and 1980 as systemic therapy due to their severe nephrotoxicity. The molecular mechanism for permeabilization by polymyxin B is thought to involve the competition for the binding of divalent cations that normally cross-bridge neighboring LPS molecules. The displacement of these stabilizing interactions leads to enhance lateral diffusion of LPS [33,35]. The resulting destabilization of the LPS layer allows the penetration of polymyxin B into the periplasm, providing essentially a "self-promoted uptake pathway" for polymyxin B to reach its target, the cytoplasmic membrane. Then, the fatty acid tail on polymyxin B allows it to permeabilize the IM, thus leading to its antibacterial action. Polymyxin B nonapeptide (PMBN) lacks the fatty acid chain, and is less bactericidal and less toxic for eukaryotic cells, but the fact that it sensitizes cells to hydrophobic antibiotics demonstrates that it retains membrane permeabilizing properties [33].

Now, faced with the continuous increase of infectious diseases involving MDR bacteria and with the scarcity of new active antibiotics, polymyxin group represents a last resort for clinicians [33,36,37]. Regarding their use in monotherapy, it must be noted that polymyxin resistance has been reported for clinical isolates although the resistant strains showed a noticeable decrease of fitness [33,38]. A possible alternate way is to combine low concentration (sub-inhibitory) of polymyxin with another clinical antibiotic to synergize its activity. Interesting results have been reported and the combination of meropenem and colistin showed increased synergic killing of Acinetobacter baumannii and P. aeruginosa [39]. Similar results have been reported with a combination of high-dose tigecycline and colistin [40]. A synergistic combination of colistin with rifampin [41] and vancomycin [42] has been reported. Moreover, significant bactericidal activity was obtained in carbapenem-resistant K. pneumoniae, A. baumannii, P. aeruginosa, and E. coli isolates using combinations of polymyxin B, doripenem, and rifampin [43]. Combination of polymyxins with these antibacterials may support a strategy for treatment of patients infected with such bacteria exhibiting resistant phenotype. The effect and kinetics reported may suggest that polymyxin may favor the entry of b-lactam molecules or other antibiotics. Polymyxin B and colistin have been also successfully used in combination with tigecycline and gentamicin against Gram-negative resistant isolates [44]. Consequently they are mentioned on the treatment of resistant bacterial infections and with association with particular antibiotics [33]. Interestingly, it has been recently demonstrated that PMBN and polymyxin B can potentialize the activity of a new family of antibacterial agents, peptide deformylase inhibitor, against Gram-negative bacteria such as E. coli, E. aerogenes, K. pneumoniae, and P. aeruginosa [45]. The increase of bacterial susceptibility is associated with the increase of the entry of new antibacterial agents obtained by the addition of sub-inhibitory concentration of polymyxin [45].

Less toxic polymyxin derivatives would be highly welcome, such as the polymyxin B non-apeptide, to be used in combination with usual antibiotics [33]. Interestingly, the group of M. Vaara has produced and characterized many polymyxin derivatives showing a significant decrease in toxicity but exhibiting chemosensitizer properties. Using sub-inhibitory concentrations, some compounds are able to increase the activity of rifampin, clarithromycin, vancomycin to E. coli, K. pneumoniae, Klebsiella oxytoca, Enterobacter cloacae, Citrobacter freundii, and A. baumannii [33,46]. In addition, a compound is effective in combination with erythromycin in an infection animal model [33]. Furthermore, it is important to mention that the use of polymyxin compounds in combination with another antibiotic may reduce the risk of resistance emergence [33].

Interestingly, some natural compounds can act as membrane permeabilizers. The terpenic compound, eugenol is the major component of clove oil from Eugenia aromatica and was reported to act primarily by disrupting the cytoplasmic membrane [47]. The combination study of eugenol and ten antibiotics against Gram-negative bacteria, E. coli, P. aeruginosa, E. aerogenes, Salmonella typhimurium and Proteus vulgaris indicated that the minimal inhibitory concentration (MIC) of the antibiotics was 5–1000-fold lower than when used alone, indicating that the combination was synergistic [48]. The enhancement of nitrocefin uptake associated with a sensitization of the microorganisms to lysis by lysozymes or detergents in the presence of eugenol demonstrated its effect on the bacterial membrane.

2.2. Destabilization of LPS barrier

An other way to circumvent the membrane barrier is to destabilize the LPS layer by using chaotropic agents or detergents that consequently facilitate the diffusion of hydrophilic compounds through the membrane lipid bilayer. Treatment by Tris/EDTA leads to massive release of LPS in the medium, and it is believed that the reduced amount of LPS in the OM leaflet is compensated by glycerophospholipids, essentially creating patches of phospholipid bilayer, which are much more permeable to lipophilic compounds [6]. A similar situation may also be found in deep rough mutants where there is a decrease in OM protein incorporation associated with the expression of defective LPS molecules generating an unbalanced ratio of LPS/phospholipids/proteins in the OM. The achieved susceptibilities become similar to those of deep rough mutants [6]. It has been demonstrated that LPS mutants are more susceptible to antibiotics. However, a major adverse effect in the use of chaotropic agent (EDTA) is associated with their noticeable toxicity on biological membrane and consequently, their use is limited to laboratory assays.

For laboratory assays including diagnostic assays, evaluation of resistance, detection of involved mechanisms and epidemiology surveys, several compounds have been used in combination with antibiotics. EDTA could be used to promote the entry of various antibiotics in resistant strains exhibiting some LPS alterations or to reduce the enzymatic activity against specific antibiotics [6,49]. EDTA can increase the susceptibility of bacteria producing metallo-b-lactamases towards b-lactams [27]. Some detergents, like Triton or SDS, deoxycholate, could be used to permeabilize the OM and in addition facilitate the entry of various antibacterial agents and subsequently increase the susceptibility [6]. Regarding the use of detergents or biocides in combination with antibiotics, the situation is especially complex and depends on the specific use (skin treatment, local decontamination, medical devices or catheters treatment).

Ceragenins are a family of bile acid derivatives that have been modified to yield an amphiphilic morphology similar to that of endogenous antimicrobial peptides [50]. With relatively low concentrations of ceragenin, a permeabilization of the OM was observed and a synergistic effect of ceragenin and erythromycin in combination was also observed [51]. In addition, a synergistic effect is observed with combination of ceragenins and host defense molecules on resistant P. aeruginosa isolates [52]. Recently, squalamine, a natural aminosterol, has been studied for its antimicrobial activity and this molecule is able to destabilize the OM of various Gram-negative bacteria [53,54]. In combination with usual antibiotics, squalamine can restore susceptibility to various antibiotic classes including b-lactams, fluoroquinolones, macrolides, phenicols, cyclines to resistant isolates that are devoid of porins [55]. This is interesting in the case of cefepime or ciprofloxacin, two antibiotics that required porins to penetrate the OM [6,8]. Neamine derivatives represent also a possibility to permeabilize bacterial

membrane. In a recent publication, Ouberai et al., clearly demonstrated that some neamine compounds are able to induced a decrease of the thickness of P. aeruginosa envelope in parallel with membrane depolarization. In addition, a binding to LPS and the induction of membrane permeabilisation is reported with some specific neamine derivatives [56].

In conclusion, these data suggest than this class of amphiphilic derivatives could be used for the development of a direct antibacterial agent, or alternatively for the production of original adjuvants that rejuvenate old antibiotic activity on MDR bacteria [57,58].

2.3. Nanoparticles and antibiotics

At this moment, new complexes using nanoparticle technology have been produced and comprise several antibacterial agents associated with nanoparticle surfaces. This new presentation of antibacterials is attractive for efficiently combating resistance associated with biofilm structures or bacterial isolates exhibiting a modified envelope that contributes to antibiotic resistance. Now, precise informations are needed to clearly identify the benefit and possible use of such approach. A serious concern associated with biomedical and surgical devices and transplants, grafts, and other invasive treatments, is that of bacterial infections and the devices colonized by bacteria may cause infection or mortality. To prevent and to combat such infections, an effective strategy is to develop original surface modification processes that provide antibacterial abilities to biomedical devices [59].

Nano silver particles comprise nano-sized structures formed from silver atoms. It is proposed that silver ions interact with main bacterial components to produce the bactericidal effect: the peptidoglycan and the membrane, DNA and proteins and especially enzymes involved in vital cellular processes such as the electron transport chain [for a review see 60]. Taking into account these destabilizing effects generated in the bacterial envelope, it may be possible to propose a combination of such new medical devices associated with usual antibiotics for the treatment of specific infectious diseases caused by resistant bacteria [61,62].

3. Blocking the efflux

Because of the clear involvement of the resistance nodulation cell division (RND) transporters in the increased frequency of MDR clinical bacteria, efflux pumps are now considered as an attractive target for the development of a combinational therapy using antibiotic/efflux pump inhibitor (EPI) as adjuvant of usual antibiotics [28]. The deciphering of structure and function, e.g., structure–activity relationship (SAR) of the RND efflux pump and its individual components is required for their rational design [28,63]. All details of interactions and processes that functionally govern and regulate the behaviour of the RND family can be useful for the development of an effective new mode of therapy. The design of active molecules capable of restoring antibiotic susceptibility in MDR pathogens is a promising alternative taking into account the scarcity of new molecules available and active against Gram-negative bacteria. These compounds must have no intrinsic antibacterial effect but induce an increase of intracellular antibiotic concentration. Some data provide some clues for the future development of distinct strategies targeting efflux pumps: alteration of pump gene expression, inhibition of membrane assembly of pump component, blocking OM exit duct, collapsing the energy driven source [21,63–65]. At the moment, efflux pump activity blockers are the main group that are described and tested on Gram-negative bacteria from both, natural and synthetic sources.

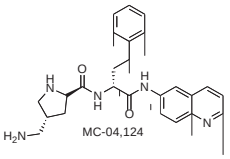
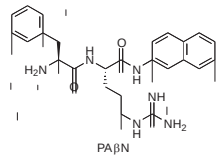
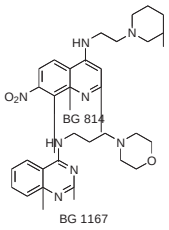
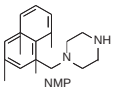
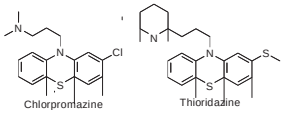
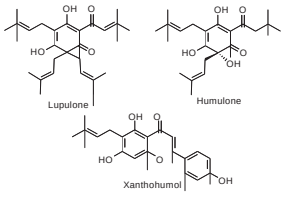
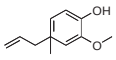
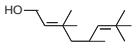
Although several families of chemical compounds that display EPIs activity have been identified, the EPIs-library is still not sufficient for a complete characterization of various aspects of multidrug resistance in Gram-negative bacteria. Many lines of evidence indicate that in case of tripartite pumps, AcrAB-TolC or MexAB-OprM, the RND protein (AcrB or MexB) is responsible for selection of a substrate to be extruded. Thus, lot of efforts are concentrated on search for compounds which tend to affine to these RND-proteins and, in this way, they could inhibit their efflux action. The 3D-structure of AcrB has been identified experimentally in several variants [66–69] for last eight years. Twenty five structures of AcrB have been submitted in Protein Data Bank (PDB) since 2002. In case of MexB, the first experimental structure has been available in PDB since 2009 [70]. It gives a great perspective to develop knowledge about the efflux mechanism and substrates binding pockets to discover new groups of efflux pump inhibitors. Nevertheless, a number of described potent EPIs is rather low, particularly, the number of inhibitors that were found basing on docking studies to the binding sites identified for the tripartite pumps within both mutagenic- and computer aided modelling studies. A natural non-selective character of the bacterial efflux proteins enables pathogens to abroad substrates with wide varieties of spatial and physicochemical properties [71]. Structure–activity relationship studies performed for families of known EPIs (Table 1), gave "step by step" new information about electron- and spatial properties of inhibitors that could be used to create pharmacophore models describing features likely necessary to interact with protein pump binding sites. The pharmacophore models could be useful for design of novel compounds with expected higher EPI-properties. Molecular modelling based pharmacophore model of Nakayama et al. [72] allowed to identify favourable features for inhibitors of MexAB-OprM in P. aeruginosa in the group of pyridopyrimidine derivatives. According to this model, four hydrophobic sites, including two sites in close neighbourhood and two peripheral sites, as well as an acidic ionisable fragment are desirable for EPI properties. The model of Nakayama allowed finding new active EPIs among pyridopyrimidine derivatives. Nevertheless, other active EPIs (Table 1) do not fit in the model as their number and position of hydrophobic sites are different, and acidic ionisable centre appears rather occasionally. A structure–activity relationship analysis which can be performed across all families of described EPIs (Table 1) gives information about structural requirements that may be desirable for interactions with efflux system of Gram-negative bacteria. The presence of various linear- or cyclic amines as well as aromatic moieties, often in neighbourhood (fused aromatic rings of naphthyl or quinoline side chains), can be noted in all representative EPIs. Amide fragments appear in several groups of EPIs, in which hydrogen bond acceptor on carbonyl oxygen may interact with amino acid residues of a binding pocket of protein pumps. Among the synthetic products with anti-efflux properties, the following chemical families stand out.

3.1. Synthetic products as efflux pump activity blockers

3.1.1. Peptidomimetics

In the case of synthetic compounds, the first recognized EPIs belong to peptidomimetic group [73], which are active against P. aeruginosa strains that overexpress the MexAB-OprM efflux pump. These compounds are the first described molecules that are able to block fluoroquinolone efflux using levofloxacin as marker [74]. Phe-Arg-b-naphthylamide (PAbN) was the first EPI-peptidomimetic, selected as a pioneer lead compound for further chemical modifications. PAbN-like peptidomimetics effectively inhibit the quinolone efflux mechanism in P. aeruginosa strain expressing MexAB-OprM, particularly levofloxacin efflux. This group of EPIs induces a competition-like process during the recognition/transport steps of antibiotic molecule [21,63]. EPI activity of PAbN was also confirmed in other Gram-negative bacteria, including E. coli, E. aerogenes, K. pneumoniae and S. enterica [21,63,75].

Table 1
Most active efflux pump blockers, with antibiotics and bacterial species in which their activity has been demonstrated.

Compound		Molecular structure	Bacterial species	Antibiotic	Reference
Synthetic products	Peptidomimetics	 MC-04,124  PAβN	P. aeruginosa, E. coli, E. aerogenes, K. pneumoniae, S. enterica	Quinolones	[63,73–80]
	Quinolines/ quinazolines	 BG 814 BG 1167	E. aerogenes	Quinolones Phenicols Cyclines Ethidium bromide	[21,63,81–83]
	Arylpiperazines	 NMP	E. coli, Acinetobacter baumanii	Fluoroquinolones	[85–90]
	Phenothiazines	 Chlorpromazine Thioridazine	B. pseudomallei, S. enterica, M. avium, M. smegmatis, E. coli	Ethidium bromide Aminoglycosides, Levofloxacin	[95–99]
Natural products	Lupulone, Humulone , Xanthohumol	 Lupulone Humulone Xanthohumol	P. mirabilis, S. marcescens, P. vulgaris	Polymyxin B	[108]
	Eugenol		E. coli, P. aeruginosa, E. aerogenes, S. typhimurium, P. vulgaris	b-Lactams, Erythromycin, Norfloxacin, Chloramphenicol, Polymyxin B, Tetracycline, Vancomycin, Rifampycin	[48]
	Geraniol		E. aerogenes, E. coli, P. aeruginosa, A. baumanii	Chloramphenicol, Norfloxacin	[109]

The mechanism proceeds using the competitive nature of PAbN with antibiotic substrates of the efflux pump system; that is while the pump preferentially pumps out PAbN, the antibiotic remains in the cell increasing its amount until the concentration required for its activity on the target is sufficient. The final effect of PAbN is to reduce resistance or completely reverse resistance to a given antibiotic to which the bacterium was initially resistant. Results of various microbiological assays suggested that PAbN may occupy an affinity site in the large substrate-binding pocket which differs from the site occupied by a given antibiotic in respect to the involved amino acid residues [76].

Derivatives molecules from PAbN-EPIs have been prepared in order to improve the stability in biological fluids, the activity and the pharmacokinetic properties of the EPI [71]. One of them, MC-04,124, is more stable than the original molecule and exhibits reduced toxicity [77,78]. These EPIs are useful to identify the presence of active efflux pump in P. aeruginosa clinical isolates [79]. From this original family of diamine EPIs several compounds have been described [80]. At this moment, these PAbN-derived EPIs are the more studied and developed family of anti-efflux compounds.

One of them is under early phase preclinical development by Mpex Pharmaceuticals. This company is now developing a fixed-combination drug product consisting of an existing antibiotic and an EPI for systemic treatment of serious hospital-acquired infections due to MDR Gram-negative bacterial pathogens such as P. aeruginosa.

3.1.2. From quinoline to quinazoline derivatives as AcrB blockers

A screening test bases on clinical isolates of E. aerogenes has allowed the identification of quinoline, quinazoline derivatives, two new classes of efflux pump blockers [81–83]. The compound structures have been selected according to their structural similarity with quinolones, a main efflux substrate. The activity of these compounds has been evaluated on a collection of MDR clinical strains

expressing various resistance mechanisms to different antibiotics including efflux pumps, altered membrane organisation, target mutations and enzymatic barriers [10]. In each class, some derivatives (Table 1) noticeably increase the susceptibility to the quinolone, phenicol and cycline antibiotics, all of which being substrates of efflux pumps of E. aerogenes [21,63,81–83]. In addition, they also increased the intracellular accumulation of radiolabelled norfloxacin or chloramphenicol [81–83]. The difference in the restoration level obtained with the various derivatives and the antibiotics tested may reflect: (i) a variation in the respective location of ligands inside the AcrB cavity such that binding of a compound interferes with the extrusion of the antibiotic; (ii) a competition between the antibiotic and derivative for the same pump active site; (iii) the compounds differ with respect to the degree of affinity for a specific internal site; and (iv) binding of the compound reduces the affinity of the antibiotic to a different site. The presence of different sites located inside the AcrB pump has been reported [12,69]. With the recent model illustrating functioning of the AcrB pump [16,19], it is possible that the same amino acid residues participated at different drug binding sites located in the pump tunnel. Moreover, several recent data on the E. coli AcrB efflux mechanism indicate that some amino acid residues are involved in the transport process to a varying extent during specific steps such as recognition, binding, transport and release [7,12,69,84]. This can explain the divergence observed in the competitive capacity of the compounds with drugs of different families (chloramphenicol, quinolones, ethidium bromide) for binding sites within the pump type (AcrB, MexB) as well as the level of expressed efflux pump activity in the tested strains [12,63]. Analyses of structure–activity relationships regarding homologous derivatives allowed the identification of pharmacophoric profile in each class of compounds.

3.1.3. Arylpiperazines

The screening of a limited library indicated that several arylpiperazines are able to block RND-type efflux pumps in E. coli [85]. The blocking activities of these compounds are modulated by the spacer length between the benzene ring and the piperazine ring as well as halogenic substitutions at the benzene ring. Among piperazine compounds, NMP (Table 1) was the most active unsubstituted arylpiperazines that increases the intracellular concentration of diverse substrates such as fluoroquinolones and fluorescent dyes [86,87]. NMP was active in several bacterial species including A. baumannii, in different Enterobacteriaceae, but less efficient in P. aeruginosa [86–90]. The mechanism of action of NMP and related compounds has not been elucidated and because of the primary activity (“serotonin agonist” properties), they are likely to be too toxic for clinical use. Nevertheless, arylpiperazine could be an interesting pharmacophoric group for other molecular structures as hydantoins [91–94] which are promising agents to combat bacterial resistance (Handzlik et al., unpublished data).

3.1.4. Phenothiazines

Phenothiazines are an other class of EPIs, or indirect effectors of antibiotic potency; derivatives such as chlorpromazine or thioridazine, have been proved to sensitize resistant bacteria to the antibiotic to which it was initially resistant [95].

For example the activity of antimicrobials against Burkholderia pseudomallei and Salmonella enterica has been increased using phenothiazines [96]. Moreover, in the presence of chlorpromazine, the efflux of ethidium bromide was decreased in the wild type parental strain as well as with thioridazine, those activity was recognized in Mycobacterium avium and smegmatis [97].

Because the primary mode of action of phenothiazines is to inhibit enzyme activities involved in the generation of metabolic energy the question of whether a similar mechanism was involved in the EPI activity of this group of agents, was of importance [98]. The effect of phenothiazine on efflux activity was demonstrated by measuring the accumulation and extrusion of ethidium bromide [99].

The activity of phenothiazines on the strain over-expressing efflux pump AcrAB E. coli has been reproduced with other Gram-negative bacteria [99]. From these preliminary data, the study on phenothiazines will be attractive for developing new physiologically relevant EPIs [63].

3.2. Natural products as a source of bacterial resistance modulators

Plants have exceptional ability to produce cytotoxic agents, and there is an ecological rationale that antimicrobial natural products should be present or synthesized de novo in plants following microbial attack to protect themselves from pathogenic microbes [100]. The scarcity of infective diseases in wild plants is in itself an indication of the successful defense mechanisms developed by them.

Numerous data are already available on Gram-positive resistance modulators in crude plant extracts or isolated from natural products [101] with the lead example of the 5^{3}-methoxyhydnocarpin produced by berberry plants that inhibits efflux of staphylococcals [102] thus allowing the active hydrophobic alkaloid berberine to reach its target [103]. In Gram-negative bacteria, despite the limited results available, some very interesting tracks are open [100].

Ethanol extract from Mentha avensis, an herbaceous plant that occurs in the whole of South America, was shown to strongly decrease resistance to gentamicin of an E. coli clinical isolate [104]. The same authors reported that extracts from Turnera ulmifolia could be used to increase the susceptibility of E. coli to aminoglycosides probably by altering efflux pump activity [105]. However, in both cases the active compound was not identified from the extracts.

Lemongrass oil (Cymbopogon citratus) or its main component, citral, were tested alone or in combination with streptomycin and kanamycin on Salmonella species. A synergy was observed for one strain of S. typhimurium in each combination between natural products and kanamycin or streptomycin. The stronger effect was observed with the combination of the essential oil and kanamycin [106]. Interestingly, citral derivatives have been showed to efficiently block NorA pump [107].

Three molecules isolated from the hop flower, lupulone (also known as beta-acid), humulone and xanthohumol were tested in combination with antibiotics on various Gram-negative bacteria. The disc diffusion assay was used to measure susceptibility to polymyxin B and zones of inhibition were compared from regular plates, to plates containing molecules at a sub-inhibitory concentration. Results indicated that in the presence of lupulone, inhibition zones around the polymyxin B discs increased for Proteus mirabilis, Serratia marcescens and P. vulgaris; the latter showing the most significant response, the inhibition zone increasing from 0.05 cm to 0.8 cm in the presence of the product [108]. Authors hypothesized that the changes in envelope permeability, which may favor the penetration or reduce the efflux, caused by the hop compounds help in overcoming the resistance associated with bacterial membrane.

The essential oil of the Corsican Helichrysum italicum (HI) exhibited a strong activity against MDR E. aerogenes clinical isolates. Moreover, the essential oil was also efficient in modulation of bacterial resistance of E. coli, P. aeruginosa and A. baumanii strains. Efflux pump mutants analysis showed that HI could modulate the AcrAB efflux mechanism and other resistance mechanisms that are produced in MDR Gram-negative bacteria. Composition determination of HI indicated that the activity described comes at least in part from geraniol, which was not described to date as modulator of antibiotic resistance [109].

4. Conclusion

Decrease of membrane permeability associated with overexpression of efflux pumps largely contribute to the MDR phenotype. Down regulation of porins synthesis is described in a large collection of resistant clinical isolates associated or not with an alteration of OM organization e.g., modification of LPS structure.

It is now admitted that the membrane-associated mechanisms of resistance represent a key step for clinical isolates and it is urgent to define new ways to circumvent these bacterial mechanisms. The re-use of polymyxins reported by several teams illustrates a possible strategy to combat MDR bacteria. With our increasing knowledge of antibiotic translocation through bacterial membrane, e.g., influx and efflux processes, in clinical isolates and the molecular details of transporter–ligand interactions, we can consider new possibilities in order to restore intracellular antibiotic concentrations. Attempts to develop original agents to combat this membrane barrier mechanism used by bacteria include the research and development of inhibitors of multidrug efflux pumps and of entry facilitators.

The variety and redundancy of membrane transporters involved in the efflux pump activity, the bacterial membrane organization and toxicity issues have hampered this. However, the recent studies focused on the specificity and mechanics (structure–activity relationships) of drug efflux may lead to the development of perhaps more effective inhibitors that exhibit a reduced toxicity associated to an efficacy in restoring the antibiotic accumulation inside the bacterium. These new agents may be able to rejuvenate the activity of "old antibiotics" against MDR bacteria. A functional, pharmaco-chemical and structural analysis of pump–substrate interactions will be engaged to decipher the molecular bases of interaction of the pump with specific ligands allowing us to develop the rational synthesis of efflux inhibitors.

Regarding molecules able to facilitate the entry of antibiotics, several main aspects must be considered, toxicity on membrane and adverse side effects on eukaryotic cells, two-step entry in Gram-negative bacteria, OM and IM, for antibiotics that must reach a cytoplasmic target (proteins, nucleic acids). The natural compounds, polycationic cyclic lipopeptides and cationic antimicrobial peptides and the new derivatives issued from polymyxin class are quite attractive but required intensive studies to determine activity, stability and toxicity. A major issue must address the pharmacokinetic and pharmacodynamic aspects of these membranotropic molecules as a therapeutic agent and their precise conditions of use.

Until now, most adjuvants or chemosensitzers have been identified through usual random screening of synthetic compounds or natural-product libraries. With the improved resolution of the 3D structures of membrane components and bacterial transporters, the dynamic simulations and molecular modelling (SAR, QSAR, HQSAR), and the recent approaches about the kinetics of transport (influx and efflux), it will be possible to design the pharmacophoric groups involved in the drug translocation. However, this original way requires studies about the penetration and membrane diffusion and also toxicity of such complex compounds.

Whatever the selected ways, favoring the entry or blocking the efflux, a functional pharmaco-chemical and biological-structural analysis of adjuvant-antibiotic interactions must be engaged to determine the best efficient combination in order to combat specific MDR bacteria and prevent the emergence of new resistances against the adjuvant action.

Regarding the future of these adjuvants in combination with antibiotics, the development and current use of b-lactamase inhibitors such as clavulanate or tazobactam is an encouraging and fruitful clinical example.

Acknowledgements

We gratefully thank Anne Favard for the preparation of this manuscript. This study was supported by the Service de Santé des Armées, by the Université de la Méditerranée, COST Action BM0701 (ATENS) and by PHC Polonium (22531ZG).

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