# Inhibitors of Bacterial Efflux Pumps as Adjuvants in Antibiotic Treatments and Diagnostic Tools for Detection of Resistance by Efflux

Françoise Van Bambeke[1,]*, Jean-Marie Pagès[2] and Ving J. Lee[3]

[1]*Unité de Pharmacologie cellulaire et moléculaire, Université catholique de Louvain, Brussels, Belgium* [2]*EA2197 Enveloppe Bactérienne, Perméabilité et Antibiotiques, Faculté de Médecine, Université de la Méditerranée, Marseille, France;* [3]*CB Research and Development, Inc. (Adesis, Inc), New Castle, DE, USA*

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**Abstract:** Active efflux is a widespread mechanism for bacterial resistance to antibiotics, which contributes to poor intrinsic susceptibility, cross-resistance to structurally diverse classes of drugs, or selection of other mechanisms of resistance. Thus, inhibition of efflux pumps appears to be (i) a promising strategy for restoring the activity of existing antibiotics, and (ii) a useful method to detect the presence of efflux determinants in clinical isolates. Structurally dissimilar classes of inhibitors have been patented in the last decade, some are analogs of antibiotic substrates [tetracyclines, quinolones or aminoglycosides] and others, new chemical entities [including substituted indoles, ureas, aromatic amides, piperidinecarboxylic acids, alkylamino- or alkoxyquinolines, peptidomimetics, and pyridopyrimidines]. Their spectrum of activity, in terms of antibiotics and bacteria, differ significantly. Narrow spectrum inhibitors are of prime interest as diagnostic tools, while broad spectrum inhibitors are expected for adjuvant therapies. Apart from (i) a peptidomimetic inhibitor of Mex pumps in *Pseudomonas aeruginosa* (MC-04,124), for which efficacy was evaluated in animal models, and (ii) a piperidinecarboxylic acid inhibitor of fluoroquinolone efflux in Gram-positive (VX-710), which was safely administered to humans, most of these products have only demonstrated their activity *in vitro,* so further investigations are needed to evaluate their clinical potential.

**Keywords:** Efflux pumps, resistance, *S. aureus, S. pneumoniae, H. influenzae, E. coli, P. aeruginosa, E. aerogenes,* reserpine, indoles, ureas, aromatic amides, piperidine-carboxylic acid derivatives, quinolines, peptidomimetics.

## GENERAL DESCRIPTION OF ANTIBIOTIC EFFLUX PUMPS IN BACTERIA AND IMPACT FOR ANTIBIOTIC TREATMENTS

Active efflux was first described in 1980, as a causative mechanism of resistance to tetracyclines [1]. It has subsequently been found to be a widespread mechanism confering to both Gram-positive and Gram-negative organisms the capacity to expel antibiotics from all the major structural classes ([2,3] for recent reviews). More recent studies, however, suggest that antibiotics are only opportunistic substrates of these physiological transporters, since efflux pumps also play a major role in the extrusion of poorly diffusible endogenous molecules [4,5] or for protection of bacteria against exogenous, potentially harmfull, diffusible substances [6,7]. In this context, antibiotics have probably only created the necessary pressure to select for efflux pump overexpression as a non-specific mechanism of resistance ([8] for a review on the regulation of the expression of efflux pumps by antibiotics and other pump substrates).

Phylogenetically, bacterial antibiotic efflux pumps belong to five superfamilies (see <http://www.biology.ucsd.edu/~msaier/transport/> for classification and [9,10] for reviews and application to antibiotic transporters), namely (i) ABC (ATP Binding Cassette), which are primary active transporters energized by ATP hydrolysis, and (ii) SMR (**S**mall **M**ultidrug **R**esistance subfamily of the DMT [**D**rug/**M**etabolite **T**ransporters] superfamily), (iii) MATE (**M**ulti **A**n**t**imicrobial **E**xtrusion subfamily of the MOP [**M**ultidrug/**O**ligosaccaridyllipid/**P**olysaccharide flippases] superfamily), (iv) MFS (**M**ajor **F**acilitator **S**uperfamily) and (v) RND (**R**esistance/**N**odulation/**D**ivision superfamily), which are all secondary active transporters driven by ion gradients. Because these pumps are presented in details in recent reviews (topology, presence in bacterial species, main substrates [2,3,10-12]), we will focus here on the elements pertinent for the present review, namely antibiotic transport in clinically-relevant pathogens. Table (**1**) lists the main transporters identified so far in frequently encountered human pathogens, together with the main antibiotic classes they transport. It clearly appears that MFS and RND are the most abundant pumps, with MFS found in both Gram-positive and Gram-negative bacteria, and characterized by a narrow spectrum (recognizing usually one, and sometimes a few, antibiotic classes), and RND found exclusively in Gram-negative and displaying an extremely wide spectrum (recognizing usually several classes of antibiotics [from 2 to 7] together with other pharmacological agents like antiseptic compounds, dyes, or detergents [11,13,14]). Of note, ABC transporters, which play a major role in drug resistance in eukaryotic cells [15], are lesser known in bacteria, and some of them are thought to play only a marginal role in resistance (like MsrD of *S. pneumoniae* [16]).

Active efflux usually confers a moderate level of resistance (1- to 64-fold increase in MIC upon expression of efflux pumps, both in laboratory mutants and clinical isolates; see [17-22] for a few examples). Nevertheless, it

*Address correspondence to this author at the Unité de Pharmacologie cellulaire et moléculaire, UCL 7370 avenue Mounier 73, 1200 Brussels, Belgium; Tel: +32-2-764.73.78; Fax: +32-2-764.73.73;
E-mail: vanbambeke@facm.ucl.ac.be

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**Table 1. Principal Efflux Pumps Expressed in Selected Human Pathogens and their Main Antibiotic Substrates (Adapted from [2] and [10])**

| Organism | (super)family | efflux pump | antibiotics | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | β-lactams | aminoglycosides | fluoroquinolones | macrolides | tetracyclines | trimetoprim | sulfamides | chloramphenicol |
| *S.aureus* | ABC | MsrA | | | | + | | | | |
| | MFS | MdeA | | | | + | | | | |
| | MFS | NorA | | | + | | | | | + |
| | | NorB | | | + | | + | | | |
| | | Tet K-L, Tet38 | | | | | + | | | |
| *S.pneumoniae* | ABC | MsrD | | | | + | | | | |
| | MFS | MefA | | | | + | | | | |
| | | MefE | | | | + | | | | |
| | | PmrA | | | + | | | | | |
| | | Tet K-L | | | | | + | | | |
| *H. influenzae* | MATE | HmrM | | | + | | | | | |
| | MFS | TetB,K | | | | | + | | | |
| | RND | AcrAB-TolC | + | | | + | | + | | |
| *E.coli* | ABC | MacAB-TolC | | | | + | | | | |
| | MATE | YdhE | | | + | | | + | | + |
| | MFS | Bcr | | | | | + | | + | |
| | | Dep | | | | | + | | | |
| | | ErmAB-TolC | | | + | | + | | | |
| | | Fsr | | | | | | + | | |
| | | MdfA | | + | + | + | + | | | + |
| | | SetA | | + | | | | | | |
| | | Tet A-E | | | | | + | | | |
| | | Ycel | | | + | | | | | |
| | | YidY | | | | | | | | + |
| | | YebQ | | | | | | + | | |
| | RND | AcrAB-TolC | + | | + | + | + | + | + | |
| | | AcrAD-TolC | | + | | | | | | |
| | | AcrEF-TolC | + | | + | + | + | + | | |
| | | YegN | | | + | | | | | |
| | SMR | ErmE | | | | + | + | | | |

**(Table 1) Contd….**

| Organism | (super)family | efflux pump | antibiotics | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | β-lactams | aminoglycosides | fluoroquinolones | macrolides | tetracyclines | trimetoprim | sulfamides | chloramphenicol |
| *P. aeruginosa* | MFS | Tet A, C, E | | | | | + | | | |
| | | CmlA | | | | | | | | + |
| | RND | MexAB-OprM | + | | + | + | + | + | + | + |
| | | MexCD-OprJ | + | | + | + | + | + | | + |
| | | MexEF-OprN | | | + | | | + | | + |
| | | MexJK-OprM | | | | + | + | | | |
| | | MexXY-OprM | | + | + | + | + | | | |
| *E. aerogenes* | MFS | CmlB | | | | | | | | + |
| | RND | AcrAB-TolC | | | + | + | + | | | + |
| | | EefABC | | | + | + | + | | | + |

markedly affects the response of bacteria to antibiotics. Potential consequences of antibiotic active efflux have been discussed extensively elsewhere ([10,11] for reviews) and can be summarized as follows:

- Apparent poor permeability of antibiotics in some bacteria has been attributed to the constitutive expression of efflux pumps, which confers a natural resistance to unrelated antibiotics. This is best exemplified in *Pseudomonas aeruginosa*, in which disruption of the gene encoding the MexB efflux pump makes the mutants hypersusceptible to chloramphenicol, fluoroquinolones, tetracyclines or β-lactams [23].
- Cross-resistance to unrelated antibiotic classes can be observed in bacteria expressing pumps with broad substrate specificity, like RND [24]. Thus, exposure to a given antibiotic may select resistance to other classes by triggering the overexpression of these pumps. Further, efflux pumps can transport antiseptic compounds, with similar consequences in terms of cross-resistance or selective pressure [13,25]. In addition, common regulators for independent mechanisms of resistance have been described, so that exposure to an antibiotic that is not subject to efflux can trigger overexpression of efflux pumps. As an example, the expression level of the marA regulator, which is involved in the genetic control of membrane permeability via porin and AcrAB-TolC efflux pump expression, can be affected by imipenem in *Enterobacter aerogenes*, so that exposure to this carbapenem, which is not a substrate for the pump, is accompanied by a loss in susceptibility to quinolones, tetracycline, and chloramphenicol [26].
- Wide spectrum or high level resistance can be observed in bacteria in which active efflux and other mechanisms of resistance function synergistically. This is exemplified in an *Escherichia coli* strain that concomitantly expresses β-lactamase and efflux pumps, and is therefore insensitive also to β-lactams resisting enzymatic hydrolysis [18]. Likewise, the combination of target mutations and of active efflux increases the level of resistance to fluoroquinolones [27].
- Selection of mutations can be favored in bacteria overexpressing efflux pumps, because antibiotic targets become exposed to subinhibitory concentrations. This has been demonstrated in *Pseudomonas aeruginosa*, in which disruption of the three main RND efflux pumps is required in order to reduce the appearance of first-step mutants in fluoroquinolone targets (from $10^{-7}$ to $< 10^{-11}$ [17]). Few epidemiological surveys, however, document the respective contribution of efflux and mutations in resistance of clinical isolates. What can be concluded at the present stage is that it is highly variable, depending on the bacteria, the antibiotic class, and the geographic area examined, as exemplified in a recent study of macrolide resistance in 8 European countries [28].

Natural genetic recombination facilitates dissemination of efflux-mediated resistance. Expression of resistance usually appears upon mutation in the corresponding regulatory system (see [3] for review) but may also occur following mutations altering substrate specificity of transporters or acquisition of mobile genetic elements expressing non-native pumps (see [29] for review). Genetic elements encoding pumps and their regulators can be located on plasmids or on conjugative or transformable transposons

[30]. Moreover, these determinants can be transferred between distant bacterial species [31].

On these bases, it is not surprising that epidemiological surveys, although often limited to specific populations or geographic areas, report on the high prevalence of efflux pumps in clinical isolates [22,28,32,33]. Accordingly, the importance of efflux as a resistance mechanism in the clinics is acknowledged in opinion and review papers [29,34-37].

For all these reasons, strategies aimed at overcoming resistance by efflux are compelling, like the combination of β-lactamase inhibitors with β-lactams to combat resistance in β-lactamase producing pathogens [38].

## STRATEGIES TO OVERCOME RESISTANCE BY EFFLUX

### 1. Bypassing Efflux Pump Mechanisms

Even though the molecular determinants responsible for the recognition of antibiotics by efflux pumps have not yet been fully elucidated, differences in transport can be observed between structural analogs within an antibiotic family. In this respect, it is interesting to note that the newer molecules developed from the main antibiotic classes are less susceptible to efflux than older ones, as demonstrated for the third and fourth generation quinolones versus first and second generation quinolones, for ketolides versus macrolides, or for glycylcyclines versus tetracyclines ([10,11] for reviews). Optimizing the structure of a molecule within an antibiotic class by taking into account susceptibility to resistance mechanisms is thus an important design element.

### 2. Biological Inhibition of Active Efflux

A first strategy to inhibit efflux pump activity could consist of blocking either the proteins themselves, using neutralizing antibodies, or the corresponding genes, by means of antisense approaches. The latter employs antisense oligonucleotides or small interfering RNA (which selectively prevent the transcription of the gene coding for the pump), or other non-traditional antisense molecules, which can interfere with the transcription or the translation of that gene of that RNA. This patented strategy was exemplified for the inhibition of the AcrAB efflux pump in *E. coli* [39], but its application could be broadened to every pump of known sequence or regulatory mechanism, or for which antibodies can be produced. The usefulness of this strategy is based on the demonstration that deletion of the *acrAB* gene in *E. coli* restores its sensitivity to a series of antibiotics [40], while a mutation in its Mar regulator has the opposite effects [41]. This approach is primarily a tool to study the role of efflux pumps in pathogens on antibiotic exposure *in vitro*, not applicable for therapeutics.

### 3. Pharmacological Inhibition of Active Efflux

A more widely exploited strategy is the development of inhibitors of efflux pumps ([42] for a recent review), which are intended for adjunctive therapy with specific antibiotics. Conceptually, pharmacological inhibition of efflux pumps can be attained by different mechanisms [43]. The dissipation of the energy gradient that drives an efflux pump is a non-specific strategy that will not be discussed here in details. Notable example is the energy decoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), used for *in vitro* studies with bacteria efflux pumps, being also extremely toxic to eukaryotic cells. The creation of a perturbation in the outer membrane channel or the assembly of the three proteins constituting the efflux system are strategies restricted to Gram-negative bacteria, where efflux pumps consist of a tripartite protein complex working in concert (the pump itself is located in the inner membrane, and is connected to a channel crossing the outer membrane by an adaptor protein; [44] for review). The induction of a flux-competition in the pump it-self is therefore probably the more general mechanism of action for pump inhibitors. At the present stage, however, few reports are available that study the mode of action of inhibitors with efflux pumps, but the situation should change in the near future, because the first crystal structures of efflux pumps were recently obtained [45].

Figures (**1**) and (**2**) show the general structure of the main classes of inhibitors that have been patented so far, and Table (**2**) lists the most active compounds from various chemotypes and their spectrum of pump inhibitory activity.

The first efflux pumps inhibitors were fortuitously discovered from existing drugs. The most popular one is reserpine (**1**) [46-48], but similar effects were described with the phenothiazines (**2**) [49], calcium antagonists (**3**) [49,50], selective inhibitors of serotonin re-uptake (**4**) [51], or proton pump inhibitors (**5**) [52,53]. A major limitation for combining these drugs with antibiotics is that they need to be used at concentrations significantly higher than that used to exert their pharmacological effects, which makes them unviable for safety reasons. Likewise, natural products-derived inhibitors, such as 5´-methoxyhydnocarpin (**6**) [54,55] have been reported ([56] for review and [55,57-60] for other examples), but their therapeutic index is sometimes questionable, and their purification, laborious and time-consuming. The convincing demonstration of the *in vitro* capacity of these two types of molecules to restore antibiotic activity in strains encoded with efflux-mediated resistance has however stimulated research for new inhibitors that are free of pharmacological activity on eukaryotic cells.

A first category of original inhibitors are chemotypes of clinically-used antibiotics, with low intrinsic antibacterial effects. Three main families have been patented so far, namely analogs of tetracyclines, aminoglycosides, and quinolones, which minimize efflux of the corresponding antibiotics.

The second category are inhibitors that are structurally unrelated to known antibiotics, and totally new entities. Some of them inhibit pumps that efflux multiple classes of antibiotics.

Based on empiric observations on the properties of these inhibitors, one can conclude the following:

- The chemical structure of the various inhibitors (Table (**2**)) has several recurrent structural features, namely (i) aromatic rings, which are present in all molecules (except aminoglycoside analogs) and ionizable moieties, which are found in many (but not all) of the putative inhibitors. This is consistent with the fact that efflux

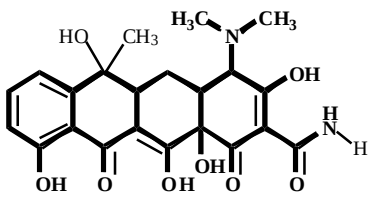
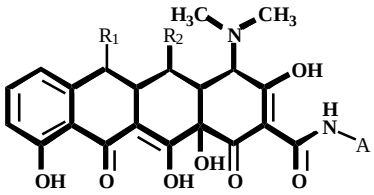
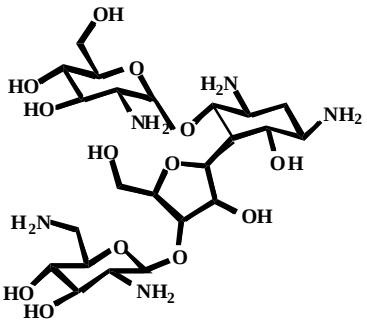
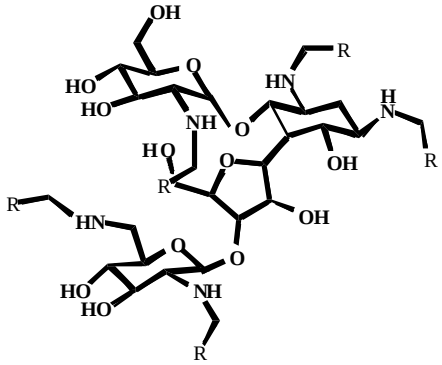
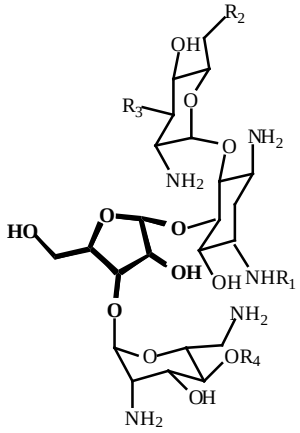
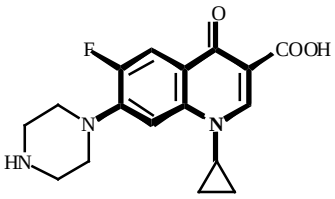
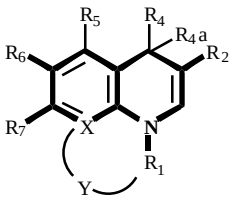
| | antibiotic | inhibitors | Patent authors; applicants [ref] |
|---|---|---|---|
| tetracyclines |  tetracycline |  | Levy; The Trustees of Tufts College [70] |
| aminoglycosides |  paromomycin |  | Nelson & Alekhsun; Paratek Pharmaceuticals, Inc. [74] |
| | |  | |
| quinolones |  ciprofloxacin |  | De Souza *et al.* ; Wockhardt Limited [75] |

**Fig. (1).** General structure of analogues of antibiotics used as inhibitors of bacterial efflux pumps. The figure shows the chemical structure of antibiotics on the left, and the general structure of inhibitors on the right. The parts common between antibiotics and inhibitors are highlighted in bold characters.

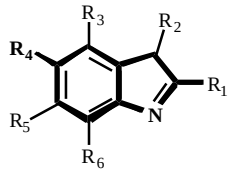
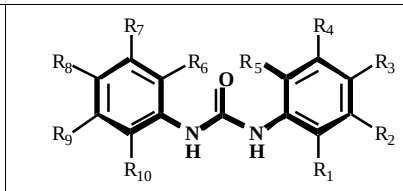
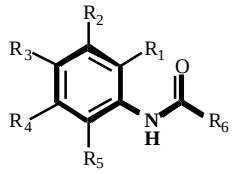
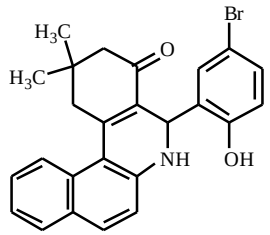
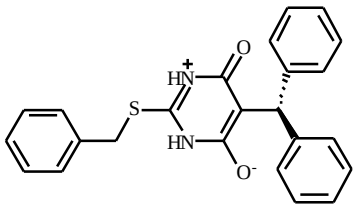
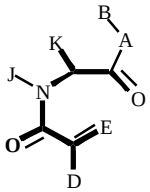
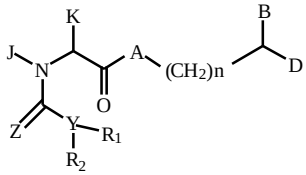
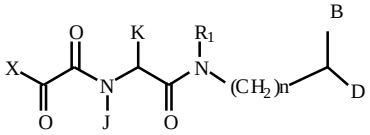
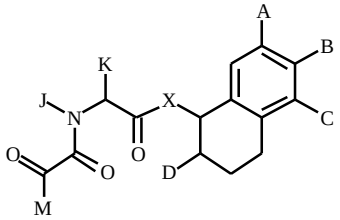
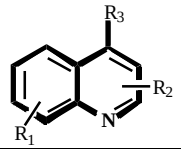
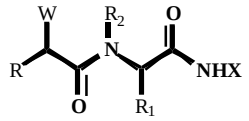
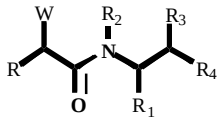
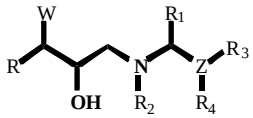
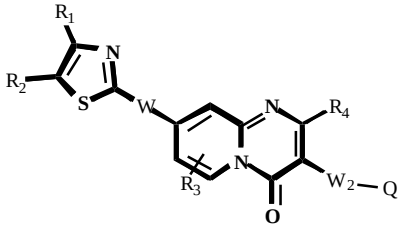
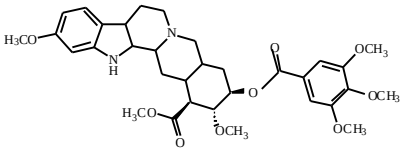
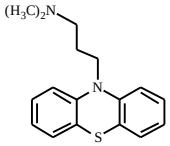
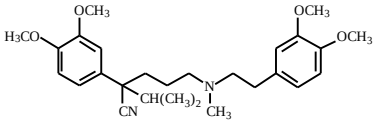
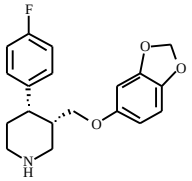
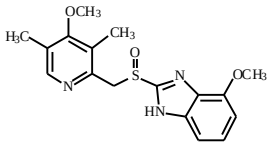
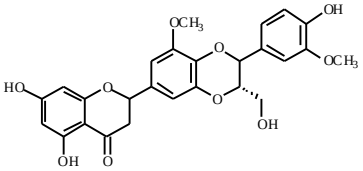
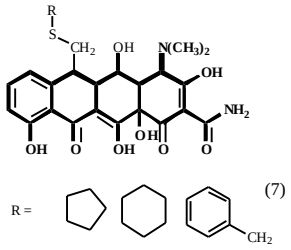
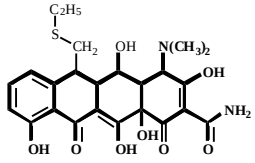
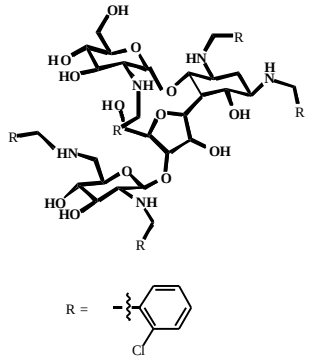
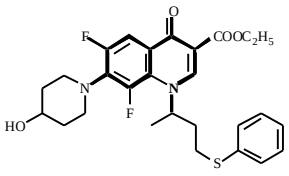
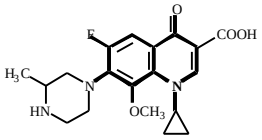
| Families patented | Patent authors; applicants [ref] |
|---|---|
|     INF 277 (**32**)  INF 392 (**33**) | Marham *et al.*; Influx, Inc. [77] |
|     | Grossman; Vertex Pharma [79] |
|  | Pages *et al.;* CNRS, INSERM, Univ. Droit, D'Economie, Sciences; Univ. de la méditerranée [91] |
|    | Chamberland *et al.*; Microcide Pharmaceuticals, Inc. [96-99] |
|  | Nakayama *et al.*; Daiichi Seiyaku Co., Essential Therapeutics Inc. [136] |

**Fig. (2).** General structure of classes of inhibitors of bacterial efflux pumps corresponding to new chemical entities that have been patented so far. Parts of the molecules appearing in bold correspond the skeleton of the inhibitors shown in Table **2**.

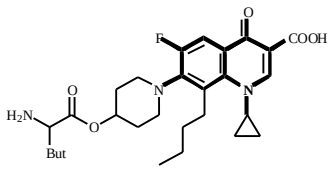
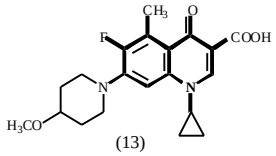
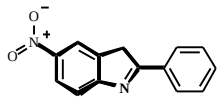
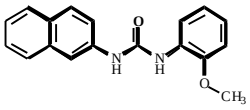
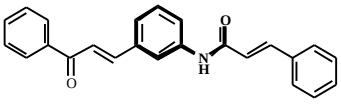
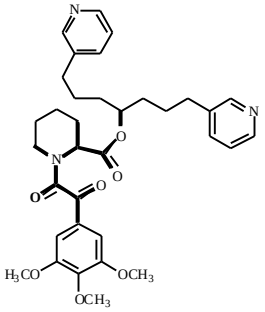
**Table 2. Most Active Inhibitors of Efflux Pumps, with Substrates and Bacterial Species in which their Activity has been Demonstrated and the Spectrum of Activity Claimed in the Corresponding Patents**

| Type of inhibitor | Ref. | Claimed spectrum of activity in corresponding patents | | Structure of the most active compounds in the series [a] | Demonstration of activity | | |
|---|---|---|---|---|---|---|---|
| | | Substrates | Bacteria | | Substrates | Bacteria | Typical active concentrations |
| **Pharmacological agents** | | | | | | | |
| alkaloids | [46,47] | No patent | |  reserpine (1) | fluoroquinolones | *S. pneumoniae* *S. aureus* | 20 µg/ml |
| phenothiazines | [49] | No patent | |  chlorpromazine (2) | tetracyclines | *E. coli* | 45 µg/ml |
| $Ca^{2+}$ antagonists | [49,50] | No patent | |  verapamil (3) | tetracyclines isoniazid | *E. coli* *M. smegmatis* | 120 µg/ml 25 µg/ml |
| phenylpiperidine selective serotonin reuptake inhibitors | [51] | No patent | |  paroxetine (4) | norfloxacin ethidium bromide tetracycline | *S. aureus* *E. coli* | 20 µg/ml |
| proton pump inhibitors | [52,53] | No patent | |  omeprazole (5) | fluoroquinolones | *S. aureus* | 100 µg/ml |
| **Natural products** | | | | | | | |
| flavonolignans | [54] | No patent | |  5´-methoxyhydnocarpin (6) | norfloxacin ethidium bromide | *S. aureus* | 10 µg/ml |

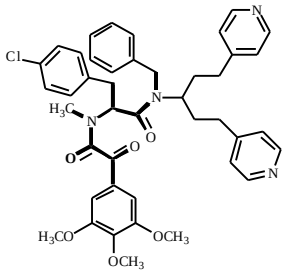
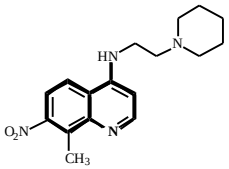
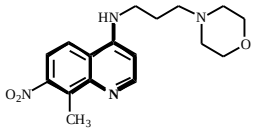
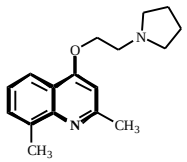
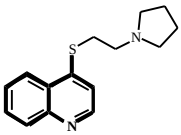
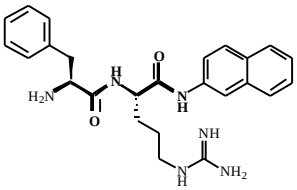
**(Table 2) Contd….**

| Type of inhibitor | Ref. | Claimed spectrum of activity in corresponding patents | | Structure of the most active compounds in the series [a] | Demonstration of activity | | |
|---|---|---|---|---|---|---|---|
| | | Substrates | Bacteria | | Substrates | Bacteria | Typical active concentrations |
| **Analogues of substrates** | | | | | | | |
| tetracyclines | [70,71] | tetracyclines | tetracycline-resistant bacteria |  (7) | tetracyclines | *S. aureus* *E. faecalis* *E. coli* | 1-2 µg/ml |
| | | | |  (8) | tetracyclines | *E. coli* | 16 µg/ml |
| Aminoglycosides | [74] | All antibiotic classes | Very wide spectrum |  (9) | tetra-cyclines, gentamicin | *H. influenzae* | ? |
| fluoro-quinolones | [75] | fluoro-quinolones macrolides tetracyclines linezolid novobiocin | Very wide spectrum |  (10) | fluoro-quinolones | *S. aureus* | < 4-20 µg/ml |
| | | | |  (11) | macrolides | *S. pneumoniae* | < 4-20 µg/ml |

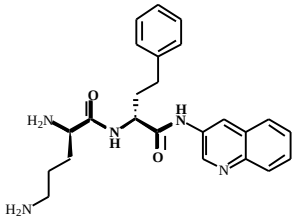
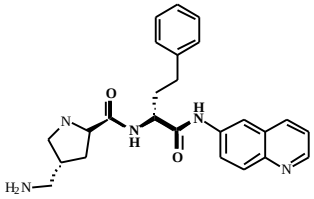
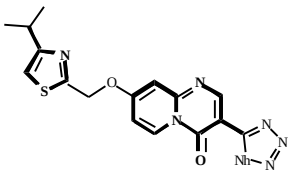
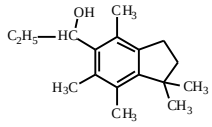
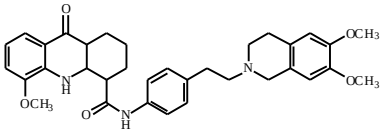
**(Table 2) Contd….**

| Type of inhibitor | Ref. | Claimed spectrum of activity in corresponding patents | | Structure of the most active compounds in the series [a] | Demonstration of activity | | |
|---|---|---|---|---|---|---|---|
| | | **Substrates** | **Bacteria** | | **Substrates** | **Bacteria** | **Typical active concen-trations** |
| | | | |  (12) | fluoro-quinolones | *E. coli* | < 4-20 µg/ml |
| | | | |  (13) | fluoro-quinolones | *P. aeruginosa* | < 4-20 µg/ml |
| **New chemical entities** | | | | | | | |
| indoles | [77,78] | fluoro-quinolones | *Staphylococci* *Streptococci* *E. faecalis* *E. coli* *P. aeruginosa* *M. smegmatis* *S. marcescens* |  IFN55 (14) | ciprofloxa-cin, ethidium bromide | *S. aureus* *S. pneumoniae* | 2.5 µg/ml |
| ureas | [77,78] | fluoro-quinolones | *Staphylococci* *Streptococci* *E. faecalis* *E. coli* *P. aeruginosa* *M. smegmatis* *S. marcescens* |  IFN271 (15) | cipro-floxacin, ethidium bromide | *S. aureus* *S. pneumoniae* | 2.5 µg/ml |
| aromatic amides | [77,78] | fluoro-quinolones | *Staphylococci* *Streptococci* *E. faecalis* *E. coli* *P. aeruginosa* *M. smegmatis* *S. marcescens* |  IFN240 (16) | cipro-floxacin, ethidium bromide | *S. aureus* *S. pneumoniae* | 2.5 µg/ml |
| piperidine-carboxylic acid derivatives | [79,90] | fluoro-quinolones macrolides rifamycins tetracyclines chloram-phenicol gentamicin linezolid penicillin amoxicilin ceftriaxone imipenem mupirocin | Very wide spectrum |  VX-710 (17) | fluoroqui-nolones gentamicin ethidium bromide novobiocin | *S. aureus* *S. pneumoniae* *E. faecalis* | 100 µg/ml |

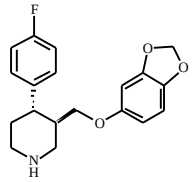
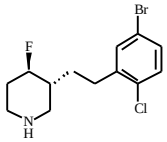
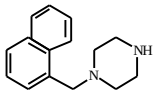
**(Table 2) Contd….**

| Type of inhibitor | Ref. | Claimed spectrum of activity in corresponding patents | | Structure of the most active compounds in the series [a] | Demonstration of activity | | |
|---|---|---|---|---|---|---|---|
| | | Substrates | Bacteria | | Substrates | Bacteria | Typical active concen-trations |
| | | | |  VX-853 (**18**) | fluoroquinolones gentamicin ethidium bromide novobiocin | *S. aureus* *S. pneumoniae* *E. faecalis* | 6 µg/ml |
| alkylamino-quinolines | [91,92] | Quinolones tetracyclines chloram-phenicol macrolides | entero-bacteriaceae |  814 (**19**) | chloramphenicol, norfloxacin, tetracycline | *E. aerogenes* | 60 µg/ml |
| | | | |  733 (**20**) | chloramphenicol | *E. aerogenes* | 330 µg/ml |
| alkoxyquinolines | [91,93] | Quinolones tetracyclines chloram-phenicol macrolides | entero-bacteriaceae |  905 (**21**) | chloramphenicol tetracycline norfloxacin | *E. aerogenes* *K. pneumoniae* | 270 µg/ml |
| thioalkoxy-quinolines | [91,94] | Quinolones tetracyclines chloram-phenicol macrolides | entero-bacteriaceae |  7d (**22**) | chloramphenicol | *E. aerogenes* | 280 µg/ml |
| peptidomimetics | [69, 92, 99,101, 131, 145-148] | All antibiotic classes | Very wide spectrum |  MC 207, 110 (**23**) | fluoroquinolones chloramphenicol erythromycin carbenicillin tetraycline ethidium bromide spectinomycin clarithromycin | *P. aeruginosa* *B. pseudomallei* | 10 µg/ml |

**(Table 2) Contd….**

| Type of inhibitor | Ref. | Claimed spectrum of activity in corresponding patents | | Structure of the most active compounds in the series [a] | Demonstration of activity | | |
|---|---|---|---|---|---|---|---|
| | | Substrates | Bacteria | | Substrates | Bacteria | Typical active concen-trations |
| | | | | | nalidixic acid<br>fluoroquinolones<br>quinolones<br>chloramphenicol | *A. baumannii*<br>*S. maltophilia*<br>*Y. enterocolitica*<br>*S. enterica*<br>*E. aerogenes*<br>*E. coli* | |
| | [102] | All antibiotic classes | Very wide spectrum |  <br>MC 02,595 (**24**) | levofloxacin | *P. aeruginosa* | 10 µg/ml |
| | [22, 103] | All antibiotic classes | Very wide spectrum |  <br>MC 04,124 (**25**) | fluoroquinolones | *P. aeruginosa* | 10 µg/ml |
| pyridopyri-midines | [133, 136] | Fluoroquinol ones β-lactams | *P. aeruginosa* (expressing MexAB OprM) |  <br>(**26**) | levofloxacin, aztreonam | *P. aeruginosa* (specific to MexAB-OprM) | 2.5 µg/ml |
| indans | [106, 107] | no patent | |  <br>Ro 07-3149 (27) | tetracyclines | *S. aureus* | |
| acridine carboxamides | [114] | no patent | |  <br>GF 120918 (**28**) | fluoroquinolones, tetracycline | *S. aureus* | 10 µg/ml |

**(Table 2) Contd….**

| Type of inhibitor | Ref. | Claimed spectrum of activity in corresponding patents | | Structure of the most active compounds in the series [a] | Demonstration of activity | | |
|---|---|---|---|---|---|---|---|
| | | Substrates | Bacteria | | Substrates | Bacteria | Typical active concentrations |
| arylpiperidines | [51] | no patent | |  NNC 20-7052 (**29**) | norfloxacin ethidium bromide tetracycline | *S. aureus* *E. coli* | 20 µg/ml |
| | [116] | no patent | |  (**30**) | linezolid | *E. coli* | 32 µg/ml |
| arylpiperazines | [118] | no patent | |  (**31**) | linezolid, levofloxacin, clarithromycin, oxacillin, rifampicin, chloramphenicol, tetracycline | *E. coli* | 50 µg/ml |

[a] parts of the molecules shown in bold correspond to the common core of the whole family of inhibitors, as illustrated in fig. (**1**) and (**2**).

pumps preferentially transport amphiphilic substrates [61] and possess affinity binding pockets presenting at their surface amino-acid side chains prone to establish hydrophobic, aromatic stacking and van der Waals interactions [62].

- Some of the inhibitors also modulate eukaryotic multidrug transporters like P-glycoprotein, MRP, or BCRP, as demonstrated for verapamil (**3**) [63], VX-710 (**17**) [64], VX-853 (**18**) [65], and GF120918 (**28**) [66,67] (note that these inhibitors are not specific for ABC transporters in bacteria as they are in eukaryotic cells; see the data shown in Table (**2**)). Since antibiotics are also substrates for eukaryotic efflux pumps ([15] for review), this property is possibly advantageous. Indeed, efflux pumps expressed by eukaryotes can modulate (i) the pharmacokinetic profile of the antibiotics (absorption, distribution, elimination), and concomitantly their serum level ([15] for review), and (ii) their cellular accumulation, which impacts their activity in intracellular infections ([68] for an example). In contrast, other inhibitors like MC 207,110 (**23**) do not interact with eukaryotic transporters [69]. This favors a specificity of action and minimizes untoward effects due to inhibition of physiological functions of eukaryotic efflux pumps.

## ANALYSIS OF THE MAIN CLASSES OF EFFLUX PUMPS INHIBITORS

### Tetracycline Analogs (See Patent [70])

Inhibitors of tetracycline efflux were identified by their ability to reduce tetracycline efflux in inverted membrane vesicles enriched in one of the efflux resistance determinants. Structure-activity relationships have shown that most effective inhibition is obtained for 6-(alkylthio)methyl-doxycycline analogs (**7,8**) [71,72]. These derivatives are usually more potent inhibitors of class A or B efflux determinants (found in *E. coli)* than of class K or L (found in Gram-positive organisms), producing synergistic effects with tetracyclines in Gram-negative, but additive effects in Gram-positive [73]. However, they show an intrinsic antibacterial activity on Gram-positive, with MIC close to those of doxycycline in non-resistant strains as well as in strains resistant by ribosomal protection (TetM) [73]. This unexpected observation suggests that, in Gram-positive, these analogs are able to inhibit the pump and also bind, probably differently than conventional tetracyclines, to the tetracycline binding site on the ribosome. This may pave the way to the design of new compounds endowed with a higher intrinsic activity, encompassing strains that are resistant due to efflux or ribosomal protection.

**Aminoglycoside Analogs (See Patent [74])**

Aminoglycosides have been historically considered as poor substrates for efflux pumps, because of their highly hydrophilic nature. Recently, they were shown to be transported by (i) a few narrow spectrum efflux pumps of the MFS superfamily, which also transport sugars, and (ii) wide spectrum efflux pumps of the RND superfamily, like the AcrAD-TolC pump of *E. coli* or the MexXY-OprM pump of *P. aeruginosa* (Table (**1**)). Accordingly, the patent claims the use of analogs (**9**) of the aminoglycoside paromomycin as inhibitors of efflux pumps, based on studies with *Haemophilus influenzae.* The analogs tested show a higher intrinsic activity (1 to 4-fold decrease in MIC) against Acr-disrupted *H. influenzae* than against the wild-type strain, suggesting a competitive mode of inhibition. These analogs also increase the susceptibility of wild-type strains and clinical isolates to gentamicin and tetracyclines. Notably, the efflux of aminoglycosides has not yet been documented (neither positively, nor negatively) in *H. influenzae.*

**Fluoroquinolone Analogs (See Patent [75])**

These modified fluoroquinolones (or ester derivatives) are able to increase the activity of these antibiotics in Gram-positive and Gram-negative organisms overexpressing well-characterized efflux pumps. Optimal targeting to a given bacterial species (or a given transporter) can be obtained by modifying the substituents in position 1, 7, or 8 (**10-13**). Quite intriguingly, some of these inhibitors also restore macrolide activity in *Streptococci* overexpressing Mef pumps. In the absence of any detailed publications on these inhibitors, it is difficult to rationalize this observation in the cited patent. Noteworthy, dimeric piperazinyl-linked fluoroquinolones display potent antibacterial activity against *S.aureus*, including resistant strains due to NorA pump activity as well as mutations in topoisomerase IV [76], inferring that they combine a high intrinsic activity and a low affinity for NorA.

**Indoles, Ureas and Aromatic Amides (See Patent [77])**

Markham *et al.* screened a library of compounds by an uptake assay for ethidium bromide in NorA-overexpressing *S. aureus*, with 399 (4 %) molecules demonstrating activity and belonging to four chemotypes, namely indoles (**14**) (note the indole moiety also present in reserpine), biphenyl ureas (**15**), aromatic amides (**16**), and molecules bearing a trichloromethylaminal group [78]. Two other active compounds (INF 277 (**32**) and INF 392 (**33**)), not structurally similar with the above chemotypes, were also mentioned in the patent (Fig. (**2**)). The broad structural diversity of inhibitors suggests that the inhibited transporters have low structural specificity for substrate/inhibitor recognition.

All active products synergize the uptake of ethidium bromide and ciprofloxacin, and also reduce the selection of resistant mutants (at least 50-fold). Their inhibitory profile typically showed activity with homologous transporters, like Bmr from *Bacillus subtilis*, and, for some of them, PmrA of *Streptococcus pneumoniae* [78]. The structural diversity of molecules showing activity increases confidence that some pharmacophores will have appropriate safety profile and can be used to construct molecules usable in adjunctive therapy. For example, leads with the trichloromethylaminal group have been abandoned [78], and INF 392 (**33**) and INF 240 (**16**) have significantly different cytotoxicity profile (INF 392 (**33**) showing the highest, and INF 240 (**16**) the lowest selectivity for bacterial cells [77]).

**Piperidine-Carboxylic Acid Derivatives (See Patent [79])**

This class of molecules was initially described [64,80] and patented [81-83] as inhibitors of P-glycoprotein and of MRP-1. One of them (VX-710 (**17**); biricodar) progressed through Phase II of clinical development [84] as adjuvant for the treatment of cancer by paclitaxel, mitoxantrone or anthracyclines [85-87]. Since its pharmacokinetic and toxicity profile in humans was already established in the above studies [88,89], it may expedite its profiling for combination use with antibiotics. Since a broad range of structural variations are disclosed in the patent (Fig. (**2**)), it is probably that molecules selective for inhibition of prokaryotic or of eukaryotic transporters can be identified in the future. Simultaneous inhibition of both eukaryotic and prokaryotic transporters is indeed disadvantageous. Dual inhibitors could alter the pharmacokinetics of antibiotics or cause toxicity when used as adjuvants to antibiotics, or, on the contrary, indirectly select bacteria acquiring resistance to them when used in combination with anticancer agents.

While the efficacy of VX-710 (**17**) and VX-843 (**18**), in combination with fluoroquinolones [90], has been demonstrated so far in Gram-positive, the patent claims encompass a range of bacteria and classes of antibiotics belonging to different classes which need further validation.

**Alkylaminoquinolines, Thioalkoxyquinolines, Alkoxyquinolines (See Patent [91])**

These compounds were found to increase the accumulation and the activity of chloramphenicol in AcrAB-TolC-positive clinical isolates of *Enterobacter aerogenes*, and were selected for their selectivity, a negligible intrinsic activity and no permeabilizing effect on the membrane [92,93]. Structure-activity relationships have demonstrated that activity of alkylaminoquinolines is optimal for derivatives with piperidino- (**19**) or morpholino- (**20**) side chain [92], and that of alkoxyquinolines (**21**), for thioethers (**22**) as compared to ethers [94]. Methylation of the pendant unit of the alkoxyquinolines further increases activity [92]. The data suggests that the alkylamino moieties on the quinoline backbone play a strategic role in recognition by the pump and competition for transport. Mallea *et al.* [92] have calculated that the maximal exclusion space of alkylaminoquinolines is 20 Å, which could fit into the central pore of the inner membrane protein AcrB, which is thought to play a major role in the transport function of the protein [95], and with the restricted region of this pore in particular [45]. This suggests that inhibition could occur either on the inner membrane protein itself, or at the inner pump-outer channel junction, where this restriction is located.

Again, additional studies are needed to determine the spectrum of activity of these inhibitors, with other clinically-relevant Gram-negative bacteria expressing broad-spectrum RND transporters.

**Peptidomimetics (See Patents [96-99])**

MC 207,110 (**23**) was selected as lead compound, after screening a library of 150K natural products and synthetic molecules, for synergism with levofloxacin towards *P. aeruginosa* [69,100]. Mechanistic studies have shown that it specifically increases the activity of antibiotics that are substrates for Mex pumps without perturbing proton gradients [101]. These studies suggest that it is also a substrate for efflux pumps, since it displays low intrinsic activity only in bacteria in which the genes coding for the main efflux pumps have been disrupted. This activity seems to be due to a disruption of membrane integrity [101]. Additional structural modifications have provided derivatives for *in vivo* evaluations. The initial goal consisted of improving the proteolytic stability of the inhibitors in biological media, which was achieved by structural permutations, including using D-amino-acids, exemplary is MC 02,595 (**24**) [102]. The second goal focused on enhancing the therapeutic indices and pharmacokinetic-pharmacodynamic profile of the molecular class for *in vivo* applications. A balance of these features is present in the conformationally-restricted analogs like MC 04,124 (**25**) [103,104]. In parallel studies, structure-activity relationships have shown that the peptidic backbone present in these three inhibitors is not essential for inhibitory activity [105].

**Other Original Derivatives (Not Patented)**

Four other structural classes of inhibitors have been reported, but no associated patents or patent applications have been cited.

Ro 07-3149 (**27**) increases the accumulation of tetracyclines in *S. aureus* by non-competitive inhibition of the TetK transporter [106]. Interestingly, it loses it activity when TetK is expressed in *E. coli*, probably due to insufficient permeability of the outer membrane of this Gram-negative to the compound [106]. In contrast with the derivatives lacking the hydroxypropyl side chain, Ro 07-3149 does not affect the energy state of the pump [107].

As VX-710 (**17**) or VX-843 (**18**), GF120918 (**28**) was first described [108], and then developed as an inhibitor of P-glycoprotein and BCRP [66,67]. It underwent Phase I studies, in combination with anthracyclines [109,110] in several animal studies, to demonstrate modulation of the pharmacokinetic profile of anticancer agents [111] and some antivirals [112,113]. It was more recently shown to also markedly increase the effectiveness of fluoroquinolones, and marginally that of macrolides and tetracyclines against *S. aureus* [114]. However, the effective concentration that modulates active transport in bacteria is higher than the human toxicity levels [115].

The arylpiperidines are topologically similar to some serotonin reuptake inhibitors (**4**). The paroxetine isomer NNC 20-7052 (**29**) is actually equipotent to paroxetine as inhibitor of MFS- (NorA and TetK) and RND-class (AcrB) pumps but much less potent as an inhibitor of serotonin reuptake [51], suggesting that stereochemistry is unimportant as far as pump inhibition is concerned and that structural congeners may combine reasonable safety profile and potency. Among them, a dihalogen substituted compound (**30**) was effective in restoring linezolid accumulation in *E. coli* [116], even though linezolid has not yet been documented as potential substrate for efflux pumps in general ([117] for a preliminary report). Similarly 1-(1-naphthylmethyl)piperazine (**31**) facilitated the accumulation of levofloxacin in *E. coli* and the activity of several antibiotics [118].

## POTENTIAL USES OF EFFLUX PUMPS INHIBITORS

The first application of these inhibitors obviously would be restoration of antibiotic activity against bacteria that encode a mechanism of resistance by efflux. Since the compelling inhibitors described herein lack intrinsic antibacterial activity, they need to be used in combination with antibiotics, similar to the β-lactamase inhibitor-β-lactam combinations. At the present time, data exists for the efficacy and safety of such combinations from animal studies. A preliminary report discusses the potentiation effect of MC 04,124 (**25**) (Table (**2**)) with levofloxacin in mouse models of *P. aeruginosa* infections (thigh infection and sepsis), and that of azithromycin in a mouse model of *E. coli* pyelonephritis [119]. Except for the above studies, interest in this strategy relies mainly on *in vitro* data demonstrating synergy between inhibitors and antibiotics. The latter is accompanied by a shift of MIC to lower values, which makes the whole population more susceptible to antibiotics (as an example, the $MIC_{90}$ of a *P. aeruginosa* population to levofloxacin shifted from 8 to 0.5 mg/L in the presence of MC-207,110 [100]). Importantly also, this synergy may reduce the selection of resistant mutants, as demonstrated for (i) reserpine and quinolones in *S. aureus* [120] and (ii) MC-207,110 and quinolones in *P. aeruginosa* ([101]; in this case, the probability of selection resistant mutants falls to a same level as upon disruption of the genes encoding efflux pumps [17]). Increasing antibiotic concentration inside bacteria will indeed contribute to bring the drug concentration above the MPC (Mutation Prevention Concentration; this concentration corresponds to the lower concentration preventing the enrichment of a culture in resistant mutants; its value varies depending on the antibiotic class and the bacteria, but is at least 5-10 times higher than the MIC (see [121] for a review of the concept)).

A question still under debate is whether efflux pumps are expressed *in vivo*. Indirect evidence exists from studies in Gram-negative bacteria. For example, *P. aeruginosa* multidrug transporters are involved in the secretion of virulence factors and quorum-sensing molecules and are therefore needed for host invasion [4]. Moreover, mechanisms of regulation are common between efflux pumps and virulence genes [122]. Interestingly enough, a recent analysis shows that a cystic fibrosis epidemic strain displays an enhanced virulence (by up-regulation of its quorum-sensing system) and an increased antimicrobial resistance associated to mutations in efflux pump genes [123]. In enteropathogens, efflux pumps are essential for survival in the gut, since they expel bile salts present in this hostile environment [7,124]. In Gram-positive organisms, in contrast, the physiological roles of efflux pumps have not yet been established. The only evidence of their potential clinical importance in the clinics is that their overexpression is evidenced in clinical isolates of Gram-positive organisms [125-127], as it is in clinical isolates of Gram-negative organisms [18,128-130].

A second application of pump inhibitors is their use as diagnostic tools. Reserpine is commonly used for Gram-positive pathogen profiling [125,127] and MC 207,110 for Gram-negatives [22,129-131], but the absence of specificity of these inhibitors does not allow for classification of the active efflux pumps. The results reported from the search of specific inhibitors (26), as done for the MexAB-OprM pump in *Pseudomonas* [132-135] (patent [136] and Fig. (**2**) and Table (**2**) for structure) are instructional. When other mechanisms of resistance are present, which mask the effect of the inhibitor, false-negative results can occur in such studies. This is particularly critical for broad-spectrum pumps in multi-resistant organisms, for which a single substrate is usually used as reporter of efflux pump activity [137].

## CURRENT & FUTURE DEVELOPMENTS

In a world of increasing bacterial resistance to antibiotics, the search of therapeutic alternatives to currently existing drugs appears as a priority. This challenge can be met in two ways [138].

The first one consists in the discovery of antibiotics directed against new pathogen targets (reviewed in [139]), which are therefore not affected by existing mechanisms of resistance. This strategy is daunting because (i) the discovery of such new entities is laborious and (ii) development of resistance to these new antibiotics is inevitable. Lessons can be learned from the post-approval events of linezolid, the only novel class of antibiotics introduced in the last decade [140,141], in which resistance was rapidly observed [see [142] for a recent survey]).

An alternative and maybe more rewarding pathway towards new antibacterial therapies, embraces the development of inhibitors of resistance mechanisms, which allows extending the utility of existing antibiotics with well known pharmacological and toxicological properties. Efflux pump inhibitors belong to this second strategy.

The present review highlights inhibitors of bacterial efflux pumps, which have shown promise *in vitro*. They can be used as diagnostic tools for detection of active efflux in pathogens as a mechanism of resistance. For this application, narrow-spectrum inhibitors will be preferred, by allowing gross identification of the transporters expressed. At the present time, however, this approach is limited to epidemiological surveys, or characterization of resistant mutants in research laboratories, while detection of resistance by efflux is not yet implemented in routine clinical laboratories. The concomitant development of genotypic methods, in combination with phenotypic methods, allows for a more precise identification of the pump [137,143] will probably be adopted in the near future.

In sharp contrast, developing combinations of efflux inhibitors with antibiotics is a continuing challenge. *A priori*, broad-spectrum inhibitors have substantial potential for clinical applications. The selection could be possibly oriented towards inhibitors targeting several pumps in a given organism (to be added to antibiotics for empiric therapy) or targeting transporters of a given class of antibiotics in different bacterial species (to be used in combined formulations). In this context, inhibitors of pump functioning may have broader spectrum than competitive inhibitors, but their use *in vivo* is unlikely because they would also affect eukaryotic transporters.

Most of the inhibitors described in this manuscript were recently tested *in vitro* by small companies or isolated laboratories, which have limited preclinical and clinical capabilities. While the major pharmaceutical firms have reduced their interest in antibacterial therapeutics [144-148], they acknowledge interest in this approach to rejuvenate the activity of current antibiotics [29,35]. Corroborating this idea, Mpex Pharmaceuticals recently licensed the Microcide Pharmaceuticals efflux portfolio, and one of the leads is in Phase Ib clinical trial as an aerosol drug candidate in cystic fibrosis (CF) patients (see “news” page on the web site of the company at <http://www.mpexpharma.com>). These encouraging news suggest the interest of extensive *in vivo* studies aimed at evaluating the pharmacological properties, safety profile, and efficacy in models of infection by resistant organisms of other efflux pumps inhibitors.

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