Inhibitors of Bacterial Efflux Pumps as Adjuvants in Antibacterial Therapy and Diagnostic Tools for Detection of Resistance by Efflux

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Abstract: Active efflux is a wide-spread 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 analogues of antibiotic substrates [tetracyclines, quinolones or aminoglycosides] and others are new chemical entities [including substituted indoles, ureas, aromatic amides, piperidinecarboxylic acids, alkylamino- or alkoxyquinolines, peptidomimetics, and pyridopyrimidines]. Their spectrum of activity, in terms of companion 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 IN ANTIBACTERIAL THERAPY

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 that confers to both Gram-positive and Gram-negative organisms the capacity to expel antibiotics from all the major structural classes ([2-4] for reviews). More recent studies, however, suggest that antibiotics are only opportunistic substrates of these physiological transporters, with

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efflux pumps also playing a major role in the extrusion of poorly diffusible endogenous molecules [5-7] and protection of bacteria against exogenous, potentially harmful, diffusible substances [8-10]. In this context, antibiotics have probably provided the necessary pressure that selects for efflux pump overexpression as a non-specific mechanism of resistance ([11] 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 [12,13] 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**ligo-saccaridyllipid/**P**olysaccharide flippases] superfamily), (iv) MFS (**M**ajor **F**acilitator **S**uperfamily) and (v) RND (**R**esistance/**N**odulation/**D**ivison superfamily), which are all secondary active transporters driven by ion gradients. Since these pumps are discussed in details in recent reviews (topology, presence in bacterial species, main substrates [2,3,13,14]), 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 is clear 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 bacteria 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 [15-17]). Of note, ABC transporters, which play a major role in drug resistance in eukaryotic cells [18], are lesser known in bacteria (MsrD and PatA/PatB in *S. pneumoniae* [19, 20]; MsrA and Vga in *S. aureus* [21-23]).

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 [24-29] for a few examples). Nevertheless, it markedly affects the response of bacteria to antibiotics. Potential consequences of antibiotic active efflux have been discussed extensively elsewhere ([13, 16] 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 [30]. This is best exemplified in *Pseudomonas aeruginosa*, in which disruption of the gene encoding the MexB efflux pump makes these mutants hypersusceptible to chloramphenicol, fluoroquinolones, tetracyclines or β-lactams [31].
- Cross-resistance to unrelated antibiotic classes can be observed in bacteria expressing pumps with broad substrate specificity, like RND [32]. 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 [15,33]. In addition, common regulators for independent mechanisms of resistance have been described, so that exposure to an antibiotic that is not subject to

Table 1. Principal Efflux Pumps Expressed in Selected Human Pathogens and their Main Antibiotic Substrates (Adapted from [3] and [13])

Organism	(Super) Family	Efflux Pump	Antibiotics								
			β-lactams	Aminoglycosides	Fluoroquinolones	Macrolides	Lincosamides / streptogramin A	Tetracyclines	Trimetoprim	Sulfamides	Chloramphenicol
S. aureus	ABC	MsrA				+					
		Vga					+				
	MFS	MdeA				+					
		NorA			+						+
		NorB			+			+			
		Tet K-L, Tet38						+			
S. pneumoniae	ABC	MsrD				+					
		PatA/PatB			+						
	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		+	+	+		+			+

(Table 1) Contd…..

Organism	(Super) Family	Efflux Pump	Antibiotics								
			β-lactams	Aminoglycosides	Fluoroquinolones	Macrolides	Lincosamides / streptogramin A	Tetracyclines	Trimetoprim	Sulfamides	Chloramphenicol
		SetA		+							
		Tet A-E						+			
		Ycel			+						
		YidY									+
		YebQ							+		
	RND	AcrAB-TolC	+		+	+		+	+	+	
		AcrAD-TolC		+							
		AcrEF-TolC	+		+	+		+	+		
		YegN			+						
	SMR	ErmE				+		+			
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			+	+		+			+

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 [34].

- 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 [29]. Likewise, the combination of target mutations and of active efflux increases the level of resistance to fluoroquinolones [35]. Combination of poor influx (due to modification of porins) and increased efflux is also responsible for a significant loss of antibiotic susceptibility [36].

- 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 $< 10^{-11}$ [28]). 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 study of macrolide resistance in 8 European countries [37].

Natural genetic recombination facilitates the dissemination of efflux-mediated resistance. The expression of resistance usually appears upon mutation(s) in the corresponding regulatory system (see [2] for review) but may also occur following mutations altering substrate specificity of transporters or acquisition of mobile genetic elements expressing non-native pumps (see [38] for review). Genetic elements encoding pumps and their regulators can be located on plasmids or on conjugative or transformable transposons [39]. Moreover, these determinants can be transferred between disparate bacterial species [40].

On these basis, 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 [27,37,41-44]. Accordingly, the importance of efflux as a resistance mechanism in the clinics is acknowledged in various review papers [30,38,45-48].

Thus, 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 [49].

STRATEGIES TO OVERCOME RESISTANCE BY EFFLUX

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 analogues 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 ([13,16] 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.

Biological Inhibition of Active Efflux

A 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* [50], 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 [51], while a mutation in its Mar regulator has the opposite effects [52]. This approach is primarily a tool to study the role of efflux pumps in pathogens on antibiotic exposure *in vitro*, not applicable for therapeutics.

Pharmacological Inhibition of Active Efflux

A more widely exploited strategy is the development of inhibitors of efflux pumps ([53,54] for recent reviews), which are intended for adjunctive therapy with specific antibiotics. Conceptually, pharmacological inhibition of efflux pumps can be attained by different mechanisms [55]. 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; [56] 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 [57-59].

Figs. (**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**) [60-62], but similar effects were described with the phenothiazines (**2**) [63], calcium channel antagonists (**3**) [63,64], selective inhibitors of serotonin re-uptake (**4**) [65], or proton pump inhibitors (**5**) [66,67]. 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. Derivatives devoid of the pharmacological activity of the parent compound are now produced, as described for inhibitors of serotonin reuptake [65,68] or of omeprazole (**6**) [69,70]. Likewise, natural products-derived inhibitors, such as 5'-methoxyhydrocarpin (**7**) [71,72] or totarol (**8**) [73] have been reported ([74] for review and [71,75-78] for other examples), but their therapeutic index is sometimes questionable, and their purification, laborious and time-consuming. Semi-synthetic derivatives of these natural

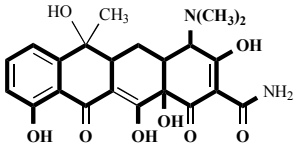
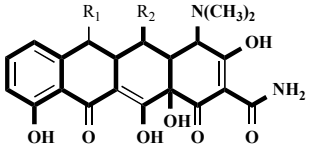
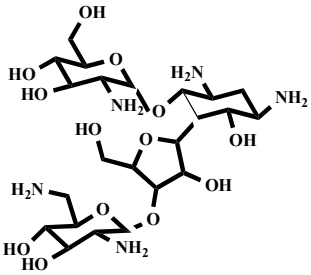
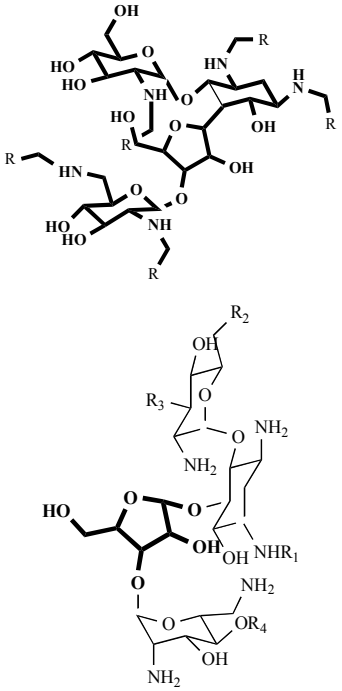
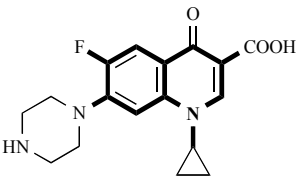
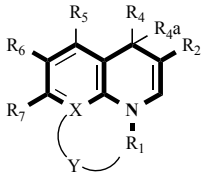
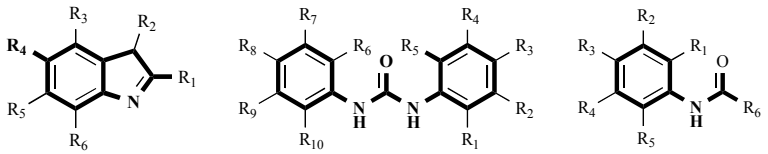
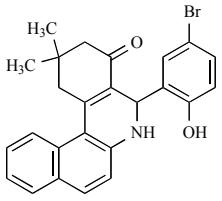
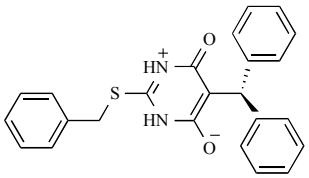
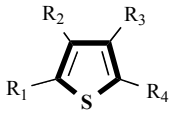
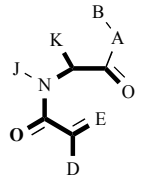
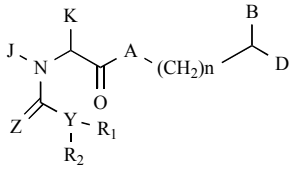
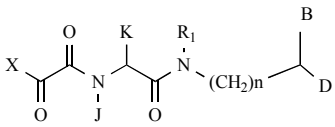
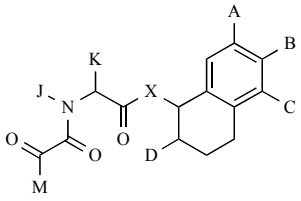
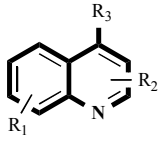
	Antibiotic	Inhibitors	Patent Authors; Applicants [ref]
Tetracyclines	 tetracycline		**Levy; The Trustees of Tufts College [93]**
Aminoglycosides	 paromomycin		**Nelson & Alekhsun; Paratek pharma-ceuticals, Inc. [97]**
Quinolones	 ciprofloxacin		**De Souza *et al.*; Wockhardt Limited [98]**

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.

products with improved activity have also been described, as exemplified for piperine analogues (**9,10**) that are 2-4-fold more potent than the parent compounds at 8-fold lower concentrations [79,80], stimulating further research in that direction [81].

Families Patented	Patent Authors; Applicants [ref]
  INF 277 (39)  INF 392 (40)	**Marham *et al.*; Influx, Inc. [100]**
	**Lemaire *et al.*; Université C. Bernard Lyon; Ecole national supérieure de chimie de Paris; CNRS [104]**
   	**Grossman; Vertex Pharma [106]**
	**Pages *et al.*; CNRS, INSERM, Univ. droit, économie, sciences; Univ. de la méditerranée [118]**

(Fig. 2) Contd…..

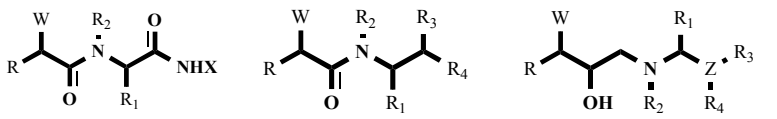
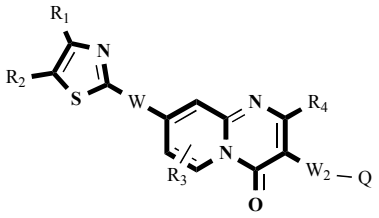
	**Chamberland *et al.*; Microcide Pharmaceuticals, Inc. [123-125,184]**
	**Nakayama *et al.*; Daiichi Seiyaku Co, Essential Therapeutics Inc. [176]**

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**.

The convincing demonstration of the *in vitro* capacity of these pharmacological agents or of natural 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 analogues of tetracyclines, aminoglycosides, and quinolones, which minimize efflux of the corresponding antibiotics.

The second category consists of 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 analogues) and ionizable moieties, which are found in many (but not all) of the putative inhibitors. This is consistent with the fact that efflux pumps preferentially transport amphiphilic substrates [82] and possess affinity binding pockets presenting at their surface amino-acid side chains prone to establish hydrophobic, aromatic stacking and van der Waals interactions [83].
- Some of the inhibitors also modulate eukaryotic multidrug transporters like P-glycoprotein, MRP, or BCRP, as demonstrated for verapamil (**3**) [84], VX-710 (**22**) [85], VX-853 (**23**) [86], and GF120918 (**34**) [87,88] (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 ([18] 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 ([18] for review), and (ii) their cellular accumulation, which impacts their activity in intracellular infections ([89-91] for examples). In contrast, other inhibitors like MC-207,110 (**28**) do not interact with eukaryotic transporters [92]. 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 Analogues (See Patent [93])

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)methyldoxycycline analogues (11,12) [94,95]. 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 [96]. 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 resistant strains due to ribosomal protection (TetM) [96]. This unexpected observation suggests that, in Gram-positive, these analogues 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.

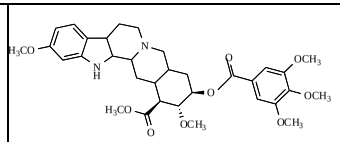
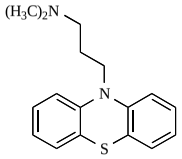
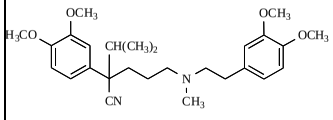
Aminoglycoside Analogues (See Patent [97])

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 analogues (**13**) of the aminoglycoside paromomycin as inhibitors of efflux pumps, based on studies with *Haemophilus influenzae.* The analogues 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 analogues 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.*

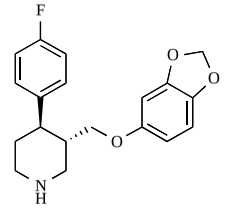
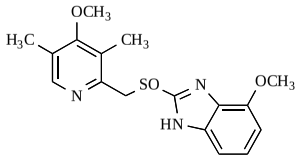
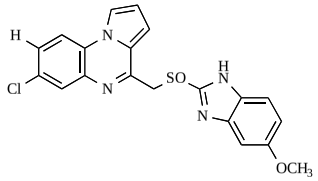
Fluoroquinolone Analogues (See Patent [98])

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 (**14-17**). 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,

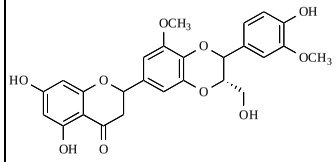
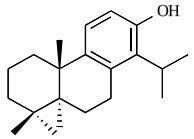
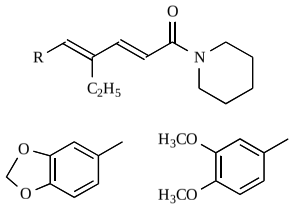
Table 2. Most Active Inhibitors of Efflux Pumps, with Substrates and Bacterial Species in which their Activity has been Demonstrated and the Spectrum of Activity Claimed in the Corresponding Patents

Type of inhibitor	Refs.	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
Pharmacological agents							
Alkaloids	[60,62]	No patent		 reserpine (1)	Fluoroquinolones	*S. pneumoniae* *S. aureus*	20 µg/ml
Phenothiazines	[63]	No patent		 chlorpromazine (2)	Tetracyclines	*E. coli*	45 µg/ml
Ca^{2+} antagonists	[63,64]	No patent		 verapamil (3)	Tetracyclines isoniazid	*E. coli* *M. smegmatis*	120 µg/ml 25 µg/ml

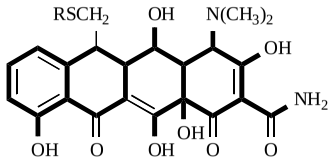
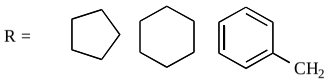
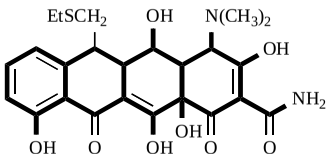
(Table 2) Contd…..

Type of inhibitor	Refs.	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
Phenylpiperidine selective serotonin reuptake inhibitors	[65]	No patent		 paroxetine (4)	Norfloxacin ethidium bromide tetracycline	*S. aureus* *E. coli*	20 µg/ml
Proton pump inhibitors	[66,67]	No patent		 omeprazole (5)	Fluoroquinolones	*S. aureus*	100 µg/ml
Pyrrolo[1,2-*a*] quinoxaline analogues of omeprazole	[69,70]	No patent		 (6)	Fluoroquinolones	*S. aureus*	128 µg/ml

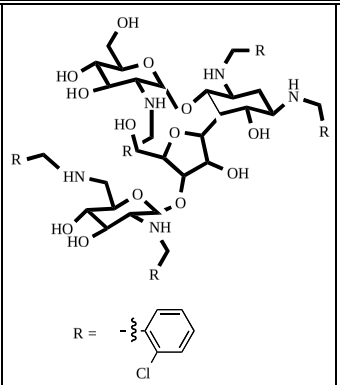
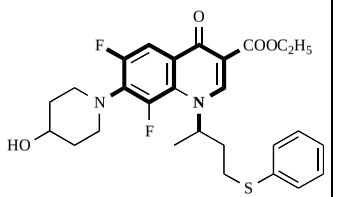
(Table 2) Contd…..

Type of inhibitor	Refs.	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
Natural products and semi-synthetic derivatives							
Flavonolignans	[72]	No patent		 5'-methoxyhydnocarpin (7)	Norfloxacin ethidium bromide	*S. aureus*	10 µg/ml
Phenolic diterpenes	[73]	No patent		 totarol (8)	Erythromycin norfloxacin tetracycline ethidium bromide	*S. aureus*	1 µg/ml
Piperine analogues	[79]	No patent		 SK20 (9) SK56 (10)	Ciprofloxacin	*S. aureus*	6.25 µg/ml

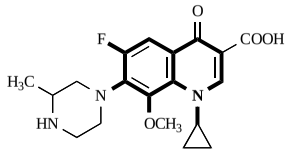
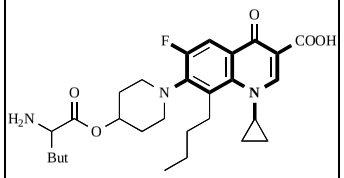
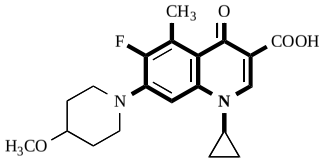
(Table 2) Contd…..

Type of inhibitor	Refs.	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
Analogues of substrates							
Tetracyclines	[93,94]	Tetracyclines	Tetracycline-resistant bacteria	 (11) R = 	Tetracyclines	*S. aureus* *E. faecalis* *E. coli*	1-2 µg/ml
				 (12)	Tetracyclines	*E. coli*	16 µg/ml

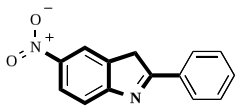
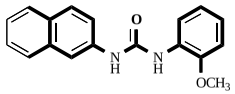
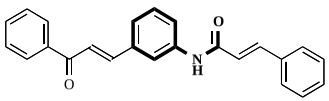
(Table 2) Contd…..

Type of inhibitor	Refs.	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
Aminoglycosides	[97]	All antibiotic classes	Very wide spectrum	 (13)	Tetracyclines, gentamicin	*H. influenzae*	?
Fluoroquinolones	[98]	Fluoroquinolones macrolides tetracyclines linezolid novobiocin	Very wide spectrum	 (14)	Fluoroquinolones	*S. aureus*	< 4-20 µg/ml

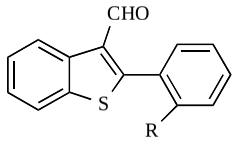
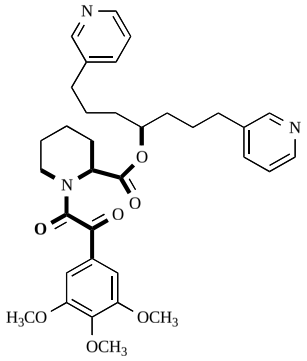
(Table 2) Contd…..

Type of inhibitor	Refs.	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
				 (5)	Macrolides	*S. pneumoniae*	< 4-20 µg/ml
				 (16)	Fluoroquinolones	*E. coli*	< 4-20 µg/ml
				 (17)	Fluoroquinolones	*P. aeruginosa*	< 4-20 µg/ml

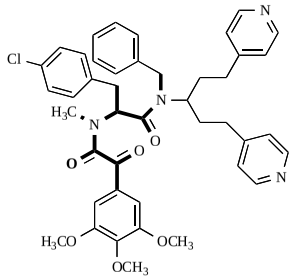
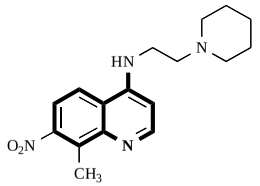
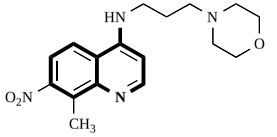
(Table 2) Contd…..

Type of inhibitor	Refs.	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
New chemical entities							
Indoles	[100,101]	Fluoroquinolones	Staphylococci Streptococci *E. faecalis* *E. coli* *P. aeruginosa* *M. smegmatis* *S. marcescens*	 IFN55 (18)	Ciprofloxacin, ethidium bromide	*S. aureus* *S. pneumoniae*	2.5 µg/ml
Ureas	[100,101]	Fluoroquinolones	Staphylococci Streptococci *E. faecalis* *E. coli* *P. aeruginosa* *M. smegmatis* *S. marcescens*	 IFN271 (19)	Ciprofloxacin, ethidium bromide	*S. aureus* *S. pneumoniae*	2.5 µg/ml
Aromatic amides	[100,101]	Fluoroquinolones	Staphylococci Streptococci *E. faecalis* *E. coli* *P. aeruginosa* *M. smegmatis* *S. marcescens*	 IFN240 (20)	Ciprofloxacin, ethidium bromide	*S. aureus* *S. pneumoniae*	2.5 µg/ml

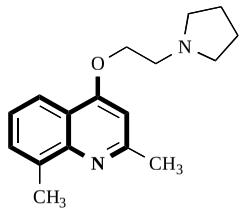
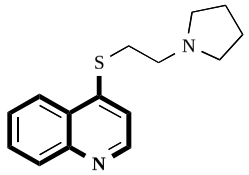
(Table 2) Contd…..

Type of inhibitor	Refs.	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
Thiophene or benzothiophene	[104,105]	Antibiotics antifungal agents	Staphylococci Streptococci Enterococci *B. subtilis* *E. coli,* *P. aeruginosa* *H. influenzae* *S. cerevisiae* *C. albicans*	 R=H; specific to NorA; R=Cl; non specific (21)	Fluoroquinolones macrolides	*S. aureus*	25 µg/ml
Piperidine-carboxylic acid derivatives	[106,117]	Fluoroquinolones macrolides rifamycins tetracyclines chloramphenicol gentamicin, linezolid penicillin, amoxicilin ceftriaxone imipenem mupirocin	Very wide spectrum	 VX-710 (22)	Fluoroquinolones gentamicin ethidium bromide novobiocin	*S. aureus* *S. pneumoniae* *E. faecalis*	100 µg/ml

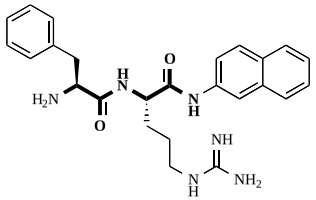
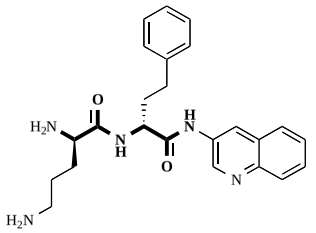
(Table 2) Contd…..

Type of inhibitor	Refs.	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 (23)	Fluoroquinolones gentamicin ethidium bromide novobiocin	*S. aureus* *S. pneumoniae* *E. faecalis*	6 µg/ml
Alkylaminoquinolines	[118,120]	Quinolones tetracyclines chloramphenicol macrolides	Entero-bacteriaceae	 814 (24)	Chloramphenicol norfloxacin tetracycline	*E. aerogenes*	60 µg/ml
				 733 (25)	Chloramphenicol	*E. aerogenes*	330 µg/ml

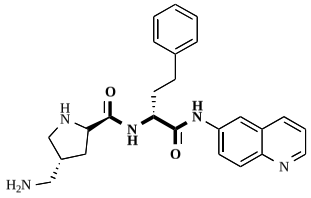
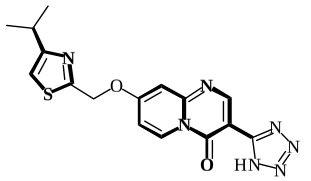
(Table 2) Contd…..

Type of inhibitor	Refs.	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
Alkoxyquinolines	[118,119]	Quinolones tetracyclines chloramphenicol macrolides	Entero-bacteriaceae	 905 (26)	Chloramphenicol tetracycline norfloxacin	*E. aerogenes* *K. pneumoniae*	270 µg/ml
Thioalkoxyquinolines	[118,121]	Quinolones tetracyclines chloramphenicol macrolides	Entero-bacteriaceae	 7d (27)	Chloramphenicol	*E. aerogenes*	280 µg/ml

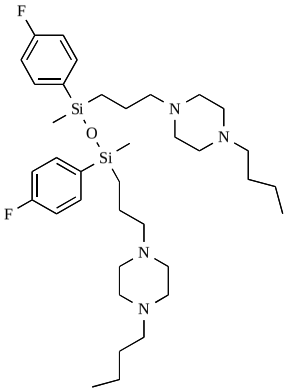
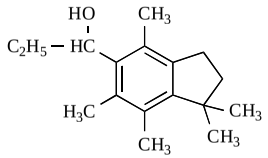
(Table 2) Contd…..

Type of inhibitor	Refs.	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**
Peptidomimetics	[92,127,171, 184-188]	All antibiotic classes	Very wide spectrum	 MC 207, 110 (28)	Fluoroquinolones chloramphenicol erythromycin carbenicillin tetracycline ethidium bromide spectinomycin clarithromycin nalidixic acid fluoroquinolones quinolones chloramphenicol	*P. aeruginosa* *B.pseudomallei* *A. baumannii* *S. maltophilia* *Y.enterocolitica* *S. enterica* *E. aerogenes* *E. coli*	10 µg/ml
	[128]	All antibiotic classes	Very wide spectrum	 MC 02,595 (29)	Levofloxacin	*P. aeruginosa*	10 µg/ml

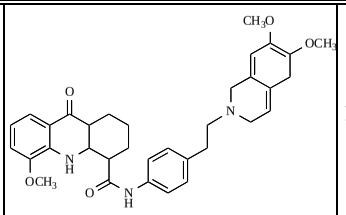
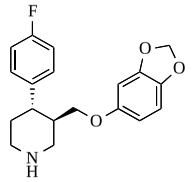
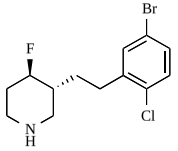
(Table 2) Contd…..

Type of inhibitor	Refs.	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
	[27,129]	All antibiotic classes	Very wide spectrum	 MC 04,124 (30)	Fluoroquinolones	*P. aeruginosa*	10 µg/ml
Pyridopyrimidines	[173,176]	Fluoroquinolones β-lactams	*P. aeruginosa* (Expressing MexAB OprM)	 (31)	Levofloxacin aztreonam	*P. aeruginosa* (Specific to MexAB-OprM)	2.5 µg/ml

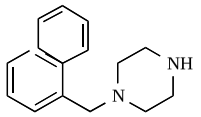
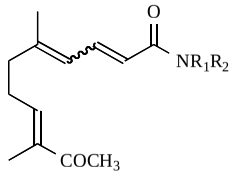
(Table 2) Contd…..

Type of inhibitor	Refs.	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
Disiloxanes	[132,134]	Patented as an adjuvant to anticancer chemotherapy		 SILA 421 (32)	(Active alone)	*M. tuberculosis*	3 µg/ml
Lindans	[135,136]	No patent		 Ro 07-3149 (33)	Tetracyclines	*S. aureus*	

(Table 2) Contd…..

Type of inhibitor	Refs.	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
Acridine carboxamides	[143]	No patent		 GF 120918 (34)	Fluoroquinolones tetracycline	*S. aureus*	10 µg/ml
Arylpiperidines	[65]	No patent		 NNC 20-7052 (35)	Norfloxacin ethidium bromide tetracycline	*S. aureus* *E. coli*	20 µg/ml
	[68,144]	No patent		 (36)	Linezolid Ethidium bromide 1-	*E. coli* *S. aureus*	32 µg/ml 6 µg/ml

(Table 2) Contd…..

Type of inhibitor	Refs.	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
Arylpiperazines	[147-150]	No patent		 NMP (37)	Linezolid levofloxacin clarithromycin oxacillin rifampicin chloramphenicol tetracycline	*E. coli* *A. baumanii* *E. aerogenes* *K. pneumoniae* *C. freundii*	50 µg/ml
9-formyl-5-methyl-deca-2,8-dienoic acid amides	[151]	No patent		 (38)	Ciprofloxacin	*S. aureus*	25 µM

[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**).

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 [99], inferring that they combine a high intrinsic activity and a low affinity for NorA.

Indoles, Ureas and Aromatic Amides (See Patent [100])

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 (**18**) (note the indole moiety also present in reserpine), biphenylureas (**19**), aromatic amides (**20**), and molecules bearing a trichloromethylaminal group [101]. Two other active compounds (INF 277 (**39**) and INF 392 (**40**)), not structurally similar with the above chemotypes, were also mentioned in the patent (Fig. **2**). Further molecules in the indole series have been produced recently, with similar activities [102,103]. The broad structural diversity of inhibitors therefore 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* [101]. 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 [101], and INF 392 (**40**) and INF 240 (**20**) have significantly different cytotoxicity profile (INF 392 (**40**) showing the highest, and INF 240 (**20**) the lowest selectivity for bacterial cells [100]).

Arylbenzo[*b*]thiophenes and Diarylthiophenes (See Patent [104])

Based on the observation that the activity of INF55 was more dependent on the 2-arylindole moiety than on the nitro substituant [101], sulfur analog of this molecule were produced, giving raise to arylbenzo[*b*]thiophenes and diarylthiophenes. These were tested for their capacity to restore ciprofloxacin activity in NorA producing and of erythromycin in MsrA producing strains of *S. aureus* and for their safety towards eucaryotic cells [105]. Most active molecules belong to the aryl benzothiophenes; the nature of the benzyl substituents affects the spectrum of activity (specificity to NorA or broader spectrum).

Piperidine-Carboxylic Acid Derivatives (See Patent [106])

This class of molecules was patented [85,107-110] as inhibitors of P-glycoprotein and of MRP-1, with VX-710 (**22**, biricodar) progressing through Phase II clinical trials [111] as adjuvant for the treatment of cancer by paclitaxel, mitoxantrone or anthracyclines [112-114]. Since its pharmacokinetic and toxicity profile in humans was already established in the above studies [115,116], 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 probable that molecules selective for inhibition of prokaryotic or 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 (**22**) and VX-843 (**23**), in combination with fluoroquinolones [117], has been demonstrated so far in Gram-positive organisms, 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 [118])

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 [119,120]. Optimal structure-activity relationships of the alkylaminoquinolines were found with piperidino- (**24**) or morpholino- (**25**) substituents [120], and that the alkoxyquinolines (**26**) and thioethers (**27**) were comparable [121]. Methylation of the pendant unit of the alkoxyquinolines further increases activity [120]. 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.,* [120] 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 [122], and with the restricted region of this pore in particular [57]. 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 [123-125])

MC-207,110 (**28**) was selected as lead compound, after screening a library of 150K natural products and synthetic molecules, for synergism with levofloxacin towards *P. aeruginosa* [92,126]. Mechanistic studies have shown that it specifically increases the activity of antibiotics that are substrates for Mex pumps without perturbing proton gradients [127]. 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 disruption of membrane integrity [127]. 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 (**29**) [128]. 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 analogues like MC 04,124 (**30**) [129,130]. In parallel studies, structure-activity relationships have shown that the peptidic backbone present in these three inhibitors is not essential for inhibitory activity [131].

Substituted Disiloxanes (See Patent [132])

SILA 421 (**38**) is a potent inhibitor of efflux pumps in cancer cells and in multidrug resistant *E. coli* [133]. Interestingly enough, it shows antibacterial activity against multidrug

resistant *Mycobacterium tuberculosis* at concentrations that are not toxic for eucaryotic cells [134]. Since this effect is obtained with SILA 421 alone, it is unlikely to result from efflux pump inhibition.

Other Original Derivatives (Not Patented)

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

Ro 07-3149 (**33**) increases the accumulation of tetracyclines in *S. aureus* by non-competitive inhibition of the TetK transporter [135]. 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 [135]. In contrast with the derivatives lacking the hydroxypropyl side chain, Ro 07-3149 does not affect the energy state of the pump [136].

Similar to VX-710 (**22**) or VX-843 (**23**), GF120918 (**34**) [137] was evaluated as an inhibitor of P-glycoprotein and BCRP [66,67]. It underwent Phase I studies, in combination with anthracyclines [138,139] in several animal studies, to demonstrate modulation of the pharmacokinetic profile of anticancer agents [140] and some antivirals [141,142]. It was more recently shown to also markedly increase the effectiveness of fluoroquinolones, and marginally that of macrolides and tetracyclines against *S. aureus* [143]. However, the effective concentration required to modulate active transport in bacteria is significantly higher than the human toxicity levels [144].

The arylpiperidines are topologically similar to some serotonin reuptake inhibitors (**4**). The paroxetine isomer NNC 20-7052 (**35**) is equipotent as paroxetine inhibiting MFS-(NorA and TetK) and RND-class (AcrB) pumps but much less potent as an inhibitor of serotonin reuptake [65], suggesting that absolute stereochemistry maybe unimportant as far as pump inhibition is concerned and that structural congeners may combine reasonable safety profile and potency. Among them, a dihalo analog (**36**) was effective in restoring linezolid accumulation in *E. coli* [145], even though linezolid has not yet been documented as potential substrate for efflux pumps in general ([146] for a preliminary report). Similarly 1-(1-naphthylmethyl)-piperazine (**37**) facilitated the accumulation of levofloxacin in *E. coli* and the activity of several antibiotics [147]. It also reverses antibiotic resistance in *A. baumanii* [148] It is however moderately active to restore fluoroquinolone activity in clinical isolates of *E. coli* and other enterobacteriaceae [149,150].

Citral-derived amides [151] were designed based on the observation that piperine [152], a major constituent of *Piper nigrum*, and semi-synthetic derivatives thereof are potent inhibitors of NorA [79-81]. The most potent molecules belong to the 9-formyl-5-methyl-deca-2,8-dienoic acid group of amides (**38**) and cause a 4-fold reduction in MIC at 25 μM [151]. This remains slightly inferior to the SK-20 (**9**) or SK-56 (**10**) analogs of piperine, which caused a 8-fold reduction in MIC at 6.25 μM [79].

Hybrid Molecules

An elegant way to facilitate the potential use of efflux pump inhibitor is to develop hybrid molecules that combine both the antibiotic moiety and an inhibitor of its efflux. *A priori*, this should permit simultaneous delivery of both molecules to the target site of infection. Two examples illustrating this strategy have been published so far.

The first one consists in a hybrid of berberine, a natural antibacterial agent, with INF55 (**18**) [153] or simplified derivatives thereof [154]. These compounds are > 300 fold more active than berberine alone against NorA overproducers; however berberine is not approved for human use. Another example are the fluoroquinolones conjugated via position 7 with an efflux pump inhibitor [155]. These compounds maintain the inhibitory activity of the inhibitor alone but show higher MICs than the parent fluoroquinolones.

Potential Uses of Efflux Pumps Inhibitors (see also [156] for Review)

The first application of these inhibitors would obviously 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 (**30**) (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 [157]. Except for the above studies, other examples of this strategy are based on *in vitro* data that demonstrate 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 (**28**) [126]). Importantly also, this synergy may reduce the selection of resistant mutants, based on the observation that resistance to quinolones by target mutation is difficult to select in strains lacking functional efflux systems [158]. A same effect was demonstrated for (i) reserpine and quinolones in *S. aureus* [159] and (ii) MC-207,110 (**28**) and quinolones in *P. aeruginosa* ([127]; in this case, the probability to select resistant mutants falls to a same level as upon disruption of the genes encoding efflux pumps [28]). Increasing antibiotic concentration in a bacteria above the MPC (Mutation Prevention Concentration), the concentration that corresponds to the minimal concentration to prevent enhancement of resistant mutants, is important. MPC values will vary depending on the antibiotic class and the bacteria, but is typically 5-10 times higher than the MIC (see [160] for a review of the concept). Of note, a recent study suggests that exposure to an efflux pump inhibitor like resepine can trigger overexpression of efflux pumps in *S. pneumoniae* [161], suggesting that resistance to inhibitors can also develop.

Considerable debate exists on 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 [6]. Moreover, mechanisms of regulation are common between efflux pumps and virulence genes [162]. Interestingly enough, 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 [163]. In enteropathogens, efflux pumps are essential for survival in the gut, since they expel bile salts present in this hostile environment [10,164]. 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 [165-167], as it is in clinical isolates of Gram-negative organisms [29,168-170].

A second application of pump inhibitors is their use as diagnostic tools. Reserpine is commonly used for Gram-positive pathogen profiling [166,167] and MC- 207,110 for Gram-negative bacteria [27,168,170,171], 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 (31), as done for the MexAB-OprM pump in *Pseudomonas* [172-175] (patent [176] 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 [177].

CURRENT AND FUTURE DEVELOPMENTS

In a world of increasing bacterial resistance to antibiotics, the search of therapeutic alternatives to currently existing drugs appears is a priority. This challenge can be met in two ways [178].

The first one consists in the discovery of antibiotics directed against new pathogen targets (reviewed in [179]), 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 [180,181], in which resistance was rapidly observed [see [182] 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 which allow gross identification of the transporters that are expressed. At the present time, phenotypic analysis approach is limited to epidemiological surveys, or characterization of resistant mutants in research laboratories; 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 [177,183] 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 [179], they acknowledge interest in this approach to rejuvenate the activity of current antibiotics [38,45]. 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>). This encouraging news suggests 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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