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Leading articles

Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy

F. Van Bambeke[1]*, Y. Glupczynski[2], P. Plésiat[3], J. C. Pechère[4] and P. M. Tulkens[1]

[1]Unité de Pharmacologie Cellulaire et Moléculaire, Université Catholique de Louvain, Brussels; [2]Laboratoire de Microbiologie, Cliniques Universitaires de Mont-Godinne, Université Catholique de Louvain, Yvoir, Belgium; [3]Laboratoire de Bactériologie, Centre Hospitalier Universitaire Jean Minjoz, Besançon, France; [4]Département de Microbiologie, Université de Genève, Geneva, Switzerland

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Originally described in bacteria, drug transporters (or efflux pumps) are now recognized as major determinants in the modulation of the accumulation and efflux of antibacterials in virtually all cell types, from prokaroytes to superior eukaryotes. Transport proteins are in fact major cellular products. Based on sequence similarities with known transporters and with proteins possessing at least two transmembrane segments, it has been calculated that 15–20% of the genome of *Escherichia coli* or of *Saccharomyces cerevisiae* may code for this type of protein.[1] At least 300 gene products are proposed to transport known substrates effectively, out of which ~20–30 transport antibiotics and other drugs.[2] Figure 1, on this basis, identifies the main groups of transporters (also referred to as superfamilies) that have been shown so far to act effectively upon antibiotics. Two of these superfamilies [major facilitator superfamily (MFS) and ATP binding cassette superfamily (ABC)] span the prokaryote–eukaryote boundary, but with specific members in each kingdom. It must be remembered, however, that most transporters have been identified only very recently, so that the discovery of many more families, with both prokaryotic and eukaryotic members, would not be surprising in the near future. Efflux pumps usually consist of a monocomponent protein with several transmembrane spanning domains (most often 12 of them). However, in Gram-negative bacteria, which are protected by an outer membrane, efflux transporters can be organized as multicomponent systems, in which the efflux pump located in the inner membrane works in conjunction with a periplasmic fusion protein and an outer membrane protein (Figure 2).[3] This first review focuses on the impact for antibiotic treatments of efflux pumps found in prokaryotes, while the companion paper[4] examines those characterized in eukaryotes.

Why antibiotic transporters?

The first description of antibiotic transporters in bacteria resulted from the study of resistance to tetracyclines in the early 1980s.[5] Soon after, several transporters were identified based on the same approach, i.e. through the unravelling of resistance mechanisms towards various classes of antibacterials, including successively the macrolides,[6] the fluoroquinolones,[7] the β-lactams[8] and, more recently, the aminoglycosides.[9] This led eventually to the concept that efflux must be considered as a common and basic mechanism of resistance, along the same lines as, and perhaps even more ubiquitous than, target modification or production of antibiotic-inactivating enzymes. The reason why antibiotics are subject to efflux may simply be that most of them share the necessary basic structural features for effective recognition. These determinants are most often the simple combination of an amphipathic character and the presence of an ionizable function. In this context, antibiotics appear as occasional substrates of transporters aimed at protecting cells from exogenous, diffusible molecules. Other examples of potentially harmful substrates include biocides[10] or bile salts for Enterobacteriaceae.[11] But efflux pumps can also be viewed as machines developed by cells to extrude poorly diffusible or toxic endogenous molecules from the cytosol, and the latter may constitute some of the natural substrates of these transporters. Using *Pseudomonas aeruginosa* PAO1 wild-type and its efflux mutants, it was shown with a model of epithelial cells and of murine endogenous septicaemia, that most mutants demonstrated significantly reduced invasiveness and decreased capacity to kill mice. Invasiveness was restored by complementation of the mutants with the wild-type genes, or

*Corresponding author. Tel: +32-2-764-73-78; Fax: +32-2-764-73-73; E-mail: vanbambeke@facm.ucl.ac.be

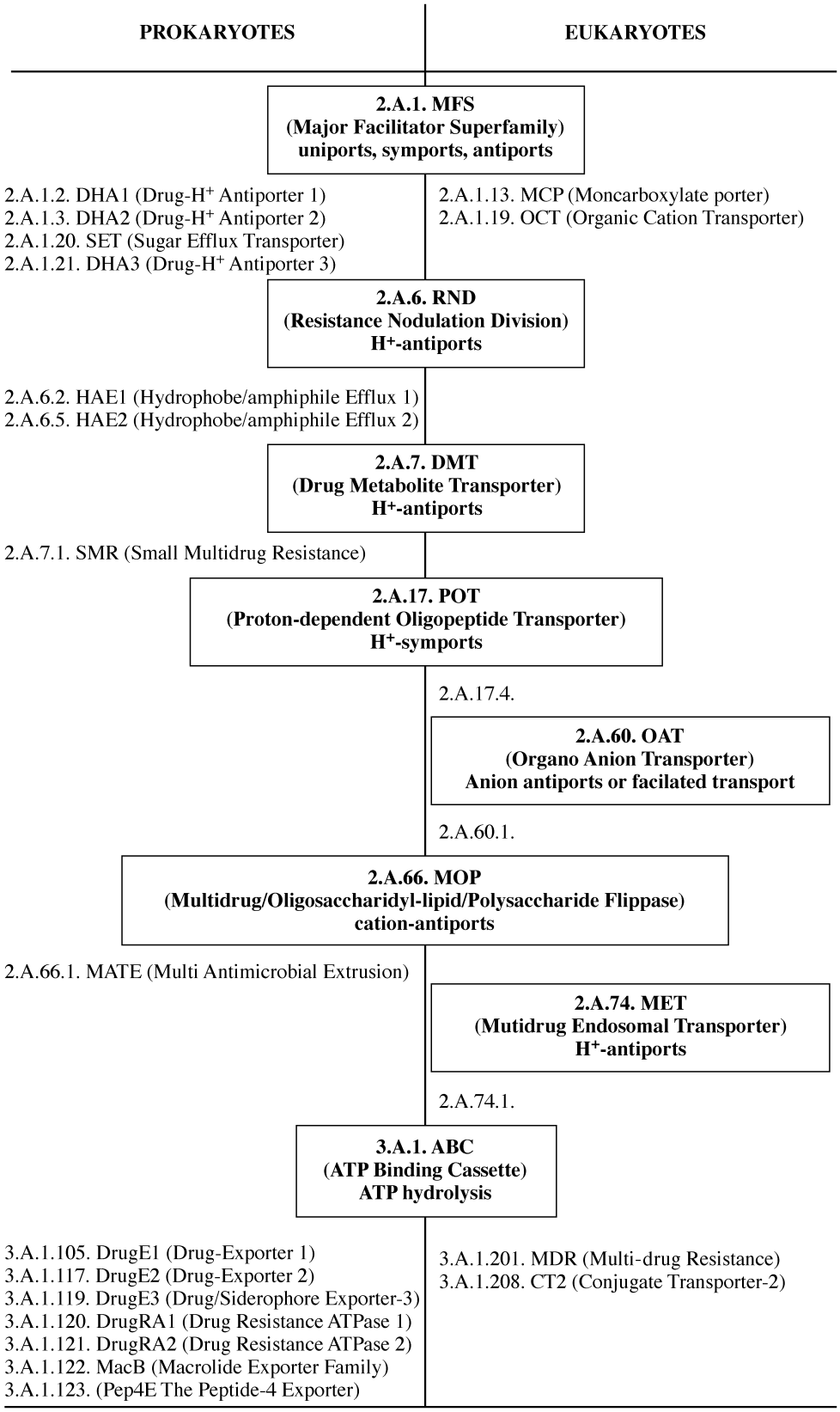

Figure 1. Occurrence and distribution of antibiotic transporters in prokaryotic and eukaryotic cells. The nomenclature is based on the classification of Saier.[2] The transporters are grouped in so-called superfamilies (three-component nomenclature; bold characters), six of which are found in prokaryotes and eukaryotes. Within each superfamily, only those families (four-component nomenclature; normal characters) transporting antibiotics are shown. OAT and MET are not found in prokaryotes.

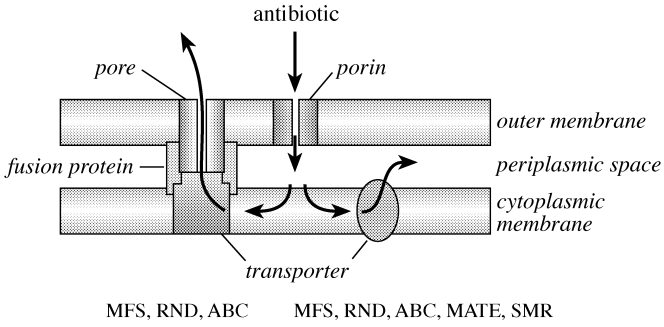

Figure 2. Topology of multicomponent (left) and monocomponent (right) efflux pumps as they can be found in the MFS, RND and ABC superfamilies and in the MFS, RND, ABC and MATE superfamilies, respectively. Multicomponent efflux pumps are specific to Gram-negative bacteria, since their particular organization allows extrusion of antibiotics directly into the extracellular medium. Arrows show directions of antibiotic transport. MFS, RND, SMR and MATE are energized by ion gradients and ABC by ATP hydrolysis.

by adding culture supernatants from epithelial cells infected with wild-type.[12] Along the same lines, the quorum-sensing auto-inducer 3-oxo-C12-homoserine lactone, which plays a key role in *P. aeruginosa* pathogenesis, is regulated by the MexAB–OprM efflux system.[13] These effects can, however, be counterbalanced by the reduced fitness that may occur as a consequence of the extrusion of bacterial metabolites,[14] or by a decreased intracellular concentration of auto-inducing virulence factors,[15] as shown in MDR-overproducing mutants of *P. aeruginosa.* Finally, combining both self-protecting and physiological roles, transporters also export bacterial toxins[16] as well as antibiotics from antibiotic producers.[17]

What are the main antibiotic transporters?

Table 1 shows, in a systematic fashion, the antibiotic transporters positively identified in some important human pathogens. For each of them, we present the necessary elements of identification and a non-exhaustive list of antibiotics transported. Many other efflux pumps are suspected to exist, based either on phylogenetic comparison with established transporters, or on phenotypical characterization (see 18 for a recent reference). Examination of Table 1 shows three major features. First, several transporters recognize very different classes of antibiotics. This holds especially true for resistance nodulation division (RND) superfamily transporters and is the basis for the cross-resistance of several bacteria to apparently structurally unrelated drugs (substituting one class of antibiotics by another will not be a satisfactory answer to resistance detected in these isolates). Secondly, a given species (typically *P. aeruginosa* and *E. coli*) may express quite different drug transporters, leading again to multiresistant phenotypes. Thirdly, a given antibiotic may be recognized by different pumps, and some classes such as tetracyclines, macrolides, fluoroquinolones and chloramphenicol appear as quasi-universal substrates. However, individual drugs, within a given class, may show specific behaviour with respect to recognition by transporters (see Table 2). This has raised passionate debate concerning apparently closely related molecules, but may simply illustrate the critical impact of apparently minor chemical modifications in the recognition process of substrates by transporters.

Impact on resistance

Generally speaking, efflux mechanisms confer a low to moderate level of resistance only (1- to 64-fold increase in MIC),[19,20] so that their clinical relevance has been questioned.[21] The following points need, therefore, to be carefully taken into consideration. First, the intrinsic (or natural) resistance of many bacteria to antibiotics depends on the constitutive or inducible expression of active efflux systems. A typical example is *P. aeruginosa*, which was thought for a long time to be poorly susceptible to a large range of antibiotics of different classes because of the relative impermeability of its outer membrane to drugs. However, the simple disruption of the gene coding for the MexB pump dramatically increases the susceptibility of *P. aeruginosa* to β-lactams, tetracyclines, fluoroquinolones and chloramphenicol.[22] Likewise, disruption of the gene coding for MdrL in *Listeria monocytogenes* causes a 10-fold decrease in the MIC of cefotaxime,[23] which suggests that the intrinsic resistance of *L. monocytogenes* to cephalosporins may be due to other mechanisms besides the existence of penicillin-binding proteins with low affinity towards this subclass of β-lactams. Finally, the poor susceptibility of *Haemophilus influenzae* to macrolides may result partly from the presence of an efflux mechanism[24] (in addition to the well known effect of acidity on the activity of macrolides, a CO_2-containing atmosphere is necessary to grow this bacteria). This suggests fresh avenues for the design of new macrolides with enhanced activity against *H. influenzae*.

Secondly, concomitant expression of several efflux pumps in a given bacterial species may lead to apparently 'high level' resistance phenotypes when considering the shared substrates. This has been observed in Gram-negative bacteria, in which the multicomponent efflux pumps of the RND superfamily transport antibiotics from the cytosolic leaflet of the inner membrane to the periplasmic space, and the single component efflux pumps of the MFS superfamily promote efflux from this space to the external medium.[25]

Thirdly, efflux may also cooperate with other resistance mechanisms to confer not only high level but also broad-spectrum resistance. For example, the high intrinsic penem resistance of *P. aeruginosa* results from the interplay between the outer membrane barrier, theactive efflux system MexAB–OprM and AmpC β-lactamase.[26] In *E. coli*, expression of

Table 1. Main efflux transporters as observed in clinically important human pathogens with their corresponding antibiotic substrates[a]

				Antibiotics																	
				β-lactams														Q			
Pathogen	Transporter	Super-family	TC number[b]	peni	ceph	carb	m-bac	inhib β-ase	FA	AG	Tet	OX	ML	SG	LM	CHL	RIF	NAL	FQ	SM	TMP
S. aureus	NorA[7]	MFS	2.A.1.2.10													+[58]			+[58]		
	TetK-L[59]	MFS	2.A.1.3.6								+[31]										
	MdeA[60]	MFS								+[60]											
	MsrA[6]	ABC	3.A.1.121.1										+[6]								
S. pneumoniae	MefE[61]	MFS											+[61]								
	PmrA[62]	MFS																	+[62]		
	TetK-L	MFS									+[31]										
Streptococcus pyogenes	MefA[63]	MFS	2.A.1.21.2										+[63]		+[63]						
L. monocytogenes	MdrL[23]	MFS		–[23]	+[23]					–[23]	–[23]		+[23]		+[23]						
	Lde[64]	MFS																	+[64]		
	TetK-L	MFS									+[31]										
Mycobacterium tuberculosis	Mmr[65]	SMR	2.A.7.1.2.										+[65]								
	TetK-L	MFS									+[31]										
	DrrB[66]	ABC	3.A.1.105.1																+[66]		
Enterococcus spp.	Mef?[67]	MFS											+[67]								
	TetK-L	MFS									+[31]										
	EmeA[68]	MFS											+[68]		+[68]				+[68]		
	Lsa[69]	ABC												+[69]	+[69]						
H. influenzae	TetB, K	MFS									+[31]										
	AcrB-like	RND											+[70]				+[70]				
Neisseria gonorrhoeae	MtrD[71]	RND	2.A.6.2.5	+[72]					+[72]		+[72]		+[72]			+[72]	+[72]				
Salmonella spp.	AcrB[73]	RND		+[74]	+[74]				+[74]		+[74]		+[74]			+[74]	+[74]	+[74]	+[74]		
	TetA-D	MFS									+[31]										
	FloR[75]	MFS														+[75]					
Shigella dysenteriae	TetA-D	MFS									+[31]										
E. coli	EmrE[76]	SMR	2.A.7.1.3								+[77]		+[77]							+[77]	
	YdhE[78]	MATE	2.A.66.1.3													+[79]			+[79]		+[79]
	TetA-E[80]	MFS	2.A.1.2.4								+[31]										
	Bcr[81]	MFS	2.A.1.2.7								+[79]									+[82]	
	MdfA[83]	MFS	2.A.1.2.19							+[79,83]	+[83]		+[83]			+[79,83]	+[83]		+[79,83]		+[79]
	YceL[84]	MFS	2.A.1.2.21																+[79]		
	YidY[84]	MFS	2.A.1.2.22													+[79]					
	EmrB[85]	MFS	2.A.1.3.2								–[85]					–[85]		+[85]	–[85]		
	YebQ[84]	MFS	2.A.1.3.17																		+[79]

	SetA[86]	MFS	2.A.1.20.1							+[87]										
	Fsr[88]	MFS	2.A.1.35.1																	+[79]
	AcrB[89]	RND	2.A.6.2.2	+[20,90]					+[72]		+[72,90]	+[91]	+[72]		+[72,90]	+[72,90]	+[90]	+[72]		+[79]
	AcrD[84]	RND	2.A.6.2.7							+[79,92]										
	AcrF[89]	RND		+[90]					+[90]		+[90]		+[90]			+[90]				
	YegN	RND	2.A.6.2.12														+[79]	+[79]		
	YhiV	RND	2.A.6.2.13										+[79]							
	MacB[93]	ABC	3.A.1.122.1										+[93]							
Stenotrophomonas maltophilia	SmeE[94]	RND								+[95]	+[95]		+[95]		+[95]			+[95]		
P. aeruginosa	CmlA[96]	MFS	2.A.1.2.3												+[96]					
	TetA,C,E	MFS									+[31]									
	MexB[97]	RND	2.A.6.2.6	+[98,99]	+[99]	+[98]	+[99]	+[100]	+[72]		+[72]		+[72]	+[101]	+[72]	+[72]		+[72]	+[72]	+[72]
	MexD[102]	RND		+[101]	+[72]	+[101]					+[72]		+[101]	+[101]	+[72]			+[72]		+[72]
	MexF[103]	RND				−[104]	−[104]	+[100]							+[72,104]			+[72,104]		+[72,104]
	MexK[105]	RND									+[105]		+[105]							
	MexY[106]	RND		+[101]	+[101]	+[101]				+[101]	+[101]		+[101]	+[101]	+[101]			+[101]		

ABC, ATP binding cassette superfamily; MATE, multi-antimicrobial extrusion; MFS, major facilitator superfamily; RND, resistance nodulation division; SMR, small multidrug resistance; peni, penicillins; ceph, cephalosporins; carb, carbapenems; m-bac, monobactams, inhib β-ase, inhibitors of β-lactamases; FA, fusidic acid; AG, aminoglycosides; Tet, tetracyclines; OX, oxazolidinones; ML, macrolides; SG, synergistins, LM, lincosamides; CHL, chloramphenicol; RIF, rifampicin; Q, quinolones; NAL, nalidixic acid; FQ, fluoroquinolones; SM, sulfamides; TMP, trimethoprim.

[a]+, occurrence; –, absence (in both cases, through functional studies).

[b]According to the classification of Saier.[2]

class-C β-lactamase confers resistance to first- and second-generation cephalosporins, and expression of pump AcrB causes resistance to most penicillins. The global result is that the organism becomes susceptible only to third- or fourth-generation cephalosporins.[20] A more indirect but probably very effective mode of cooperation is exemplified by the combination of DNA gyrase and/or topoisomerase IV mutation and efflux in the development of resistance to fluoroquinolones. Whereas single target mutations confer only a low level of resistance, the reduction in the intrabacterial concentration of fluoroquinolones through expression of one or several efflux pumps may result in MICs exceeding breakpoints (see 27 for a recent example). Thus, resistant strains from clinical sources[28] often display a combination of multiple mutations in the target genes and overexpression of efflux transporters.[29] More critically, the exposure of the targets to insufficient drug concentrations will favour the selection of mutants. Thus, constitutive expression of efflux pumps acting on fluoroquinolones probably explains the high frequency of mutations leading to resistance in Gram-negative bacteria. This has been particularly well demonstrated for levofloxacin and *P. aeruginosa*. Disruption of the genes of three RND pumps not only brings the MIC from 0.25 to <0.02 mg/L, but, most strikingly, reduces the frequency of appearance of first-step mutants from 2×10^{-7} to $<10^{-11}$.[19] It must, however, be emphasized that all pumps must be inactivated simultaneously to obtain such an effect, since the lack of activity of one can be easily compensated for by overexpression of others with overlapping spectra.[30]

Fourthly, antibiotics can serve as inducers and regulate the expression of some efflux pumps at the level of gene transcription or mRNA translation, by interacting with regulator systems.[31] Transporters may also become overexpressed as a result of mutations occurring in these regulators[3] (this mechanism may be the predominant one for resistance of *P. aeruginosa* to fluoroquinolones in cystic fibrosis patients).[32] More importantly, global regulation may be involved,[33] causing the overexpression of several independent genes (regulons).[34] For instance, the *mar* regulon in *E. coli* may control not only the expression of AcrB, but probably also that of other drug-specific transporters and porins, together with numerous other proteins involved in stress responses (see 35 and the references cited therein). Worryingly, mutations in regulator genes might even result in constitutive expression of several efflux pumps, causing multiple resistance.

Fifthly, resistance by efflux can be easily disseminated. In several cases, the genetic elements encoding efflux pumps and their regulators are located on plasmids (such as Tet transporters in Gram-positive bacteria), or on conjugative or transformable transposons located either on plasmids (Tet transporters also, but in Gram-negative bacteria), or in the chromosome (e.g. *mef* genes in *Streptococcus pneumoniae*).[36]

Table 2. Relative affinities of antibiotics for efflux pumps

Antibiotic class	Affinity for efflux pumps			References
	high	variable[a]	low	
Penicillins[b]	nafcillin, cloxacillin, penicillin G		carbenicillin	74
Cephalosporins[b]	cefalotin, cefotaxime, ceftriaxone		cefazolin, cephaloridin	74
Carbapenems	meropenem	imipenem		98
Macrolides	14- and 15-membered		16-membered, ketolides	107–109
Tetracyclines	tetracycline	minocycline	glycylcyclines[c]	31,110,111
(Fluoro)quinolones	ciprofloxacin, norfloxacin	ofloxacin, levofloxacin	cinafloxacin, gatifloxacin, gemifloxacin, moxifloxacin,[d] garenoxacin	19,112–115

[a]Depending on the efflux pump.
[b]Ranking corresponding to the degree of lipophilicity of the side chain.
[c]Low affinity substrate of MexD in *P. aeruginosa* and AcrB and AcrF in *E. coli*.[116,117]
[d]Low affinity substrate of a still unidentified efflux transporter in *S. aureus*.[118]

Importantly, efflux-mediated resistance mechanisms can spread between phylogenically very distant species. This is exemplified by the macrolide-mediated efflux, which has moved not only among streptococci but also to Gram-negative bacteria.[37] Co-transfer with genes for other resistance mechanisms may also take place if these are present together on large mobile genetic elements.

Strategies for the future

Efflux mechanisms should now be taken fully into account in the evaluation of new antibiotics as well as for the future of chemotherapy in the long term. At the level of the clinical microbiology laboratory, suspicion of efflux as a cause of resistance ideally should be confirmed either phenotypically or genotypically so that cross-resistances during therapy can be anticipated. However, phenotypic characterization of efflux may prove difficult or impossible in multiresistant isolates that have several mechanisms. The clear-cut situation of macrolides and streptococci, in which efflux-based resistance is limited to 14- and 15-membered macrolides,[38] may be unique. Conversely, demonstrating resistance to a large variety of unrelated agents can suggest the presence of wide-spectrum transporters (see 39 for an example with clinical isolates). Unfortunately, several of the antibiotic substrates recognized by many efflux pump systems (such as chloramphenicol, tetracyclines or trimethoprim) are no longer tested in clinical laboratories because of lack of immediate therapeutic interest. More genotypic characterization could yield important information for surveillance studies. For instance, PCR has been used successfully to identify unambiguously efflux-based resistance mechanisms to macrolides,[40] tetracyclines[41] and fluoroquinolones,[29] and fingerprinting has allowed the spread of the corresponding resistant clones to be followed.[32] Further development of new genotypic tests to improve the characterization of efflux mechanisms in clinical isolates would be welcome in this context (see 42 for first attempts in this direction).

Moving to the evaluation of new antibiotics, exploring their potential recognition by typical efflux transporters must now be in the forefront of their pre-clinical and clinical assessment. All other pharmacological and toxicological properties being equal, the aim here will be to favour the selection of derivatives that are poor substrates of the efflux pumps and that do not induce their overexpression. Table 2 illustrates the relative affinity of current and newly introduced antibiotics for efflux transporters, and reveals that most recent antibiotics in each class are less well recognized than those that are older. The development of pump inhibitors as adjuvant therapy also represents an interesting area for drug discovery, similar to how β-lactamase inhibitors brought new life to β-lactams.[43] This approach, however, appears very challenging because of potential effects on efflux transporters also present in eukaryotic cells. It must be emphasized that most inhibitors currently available display strong pharmacological activities in eukaryotic cell systems and are therefore unusable in clinical practice. Typical examples include reserpine,[44] omeprazole,[45] phenothiazines,[46] sertraline,[47] verapamil[48] and siderophores.[49] It is therefore essential to develop molecules not only designed specifically to inhibit prokaryotic transporters, but also with some distance from molecular structures close to those of drugs. Another difficulty is that the pharmacokinetic/dynamic properties of the pump inhibitors will need to match closely those of the companion antibiotic. Only three main categories of compound have been uncovered so far.[50] The first group comprises compounds specifically raised against the Tet transporters for tetracyclines (namely tetracycline derivatives substituted in position 13 and probably acting as

competitive inhibitors,[51] and a non-competitive inhibitor built on an indan nucleus).[52] The second group is represented by a family of flavonolignans derived from a natural alkaloid from *Onopordon corymbosum*.[53] These molecules inhibit the NorA transporter of *Staphylococcus aureus*. As flavones are also known to inhibit the eukaryotic P-glycoprotein, considerable lead optimization studies will be needed to obtain safe compounds. The third category is represented by peptides acting upon the RND transporters of *P. aeruginosa*.[54] These inhibitors selectively improve the activity of antibiotics that are substrates of the MexB efflux pump (quinolones, macrolides, chloramphenicol, and to a lesser extent, tetracycline or carbenicillin),[55] which suggests a high level of specificity.[56] They do not interact with the eukaryotic P-glycoprotein. A desirable step forward would be to consider the design of wider spectrum inhibitors acting on pumps present in both Gram-positive and Gram-negative bacteria and belonging to different phylogenetic families. Prudent use of these inhibitors will, however, be essential, to avoid fast emergence of resistance to them, which will most probably also emerge. Laboratory mutants of *Bacillus subtilis* have already been isolated with resistance to the inhibitory activity of reserpine.[57]

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