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COMMENTARY

Antibiotic Efflux Pumps

Françoise Van Bambeke,† Elisabetta Balzi‡§ and Paul M. Tulkens**

*Unité de Pharmacologie Cellulaire et Moléculaire, Université Catholique de Louvain, Brussels; and ‡Unité de Biochimie Physiologique, Université Catholique de Louvain, Louvain-la-Neuve, Belgium

ABSTRACT. Active efflux from procaryotic as well as eucaryotic cells strongly modulates the activity of a large number of antibiotics. Effective antibiotic transport has now been observed for many classes of drug efflux pumps. Thus, within the group of primary active transporters, predominant in eucaryotes, six families belonging to the ATP-binding cassette superfamily, and including the P-glycoprotein in the MDR (Multi Drug Resistance) group and the MRP (Multidrug Resistance Protein), have been recognized as being responsible for antibiotic efflux. Within the class of secondary active transporters (antiports, symports, and uniports), ten families of antibiotic efflux pumps have been described, distributed in five superfamilies [SMR (Small Multidrug Resistance), MET (Multidrug Endosomal Transporter), MAR (Multi Antimicrobial Resistance), RND (Resistance Nodulation Division), and MFS (Major Facilitator Superfamily)]. Nowadays antibiotic efflux pumps are believed to contribute significantly to acquired bacterial resistance because of the very broad variety of substrates they recognize, their expression in important pathogens, and their cooperation with other mechanisms of resistance. Their presence also explains high-level intrinsic resistances found in specific organisms. Stable mutations in regulatory genes can produce phenotypes of irreversible multidrug resistance. In eucaryotes, antibiotic efflux pumps modulate the accumulation of antimicrobials in phagocytic cells and play major roles in their transepithelial transport. The existence of antibiotic efflux pumps, and their impact on therapy, must now be taken fully into account for the selection of novel antimicrobials. The design of specific, potent inhibitors appears to be an important goal for the improved control of infectious diseases in the near future. BIOCHEM PHARMACOL **60**;4:457–470, 2000. © 2000 Elsevier Science Inc.

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Biological membranes most likely appeared very early on during evolution to isolate hydrophilic microdomains from the surrounding medium, allowing catalyzed reactions to occur in an efficient manner. Biomembranes, accordingly, constitute efficient barriers towards hydrophilic molecules, most of which can penetrate cells only by specific inward transport systems or find their entry restricted to the endocytic pathway. *A contrario*, biomembranes are easily crossed by amphiphilic compounds, since these are able to diffuse through both the hydrophilic and the hydrophobic domains of the bilayer. Therefore, it is not surprising that mechanisms were devised, also very early, to protect cells from the disordered invasion by amphiphilic molecules, many of which are endowed with biological activities leading to potentially harmful effects. A major mechanism in this respect is constituted by active outward transport. Although efflux systems have been known for many years, their importance, both in terms of number and variety of substrates, has become clearly recognized only very recently. Drugs are often amphiphilic, whether by selection or by design, ensuring their wide tissue distribution and/or their penetration into membrane-protected compartments. Therefore, it comes as no surprise that many drugs should fall into this category of exogenous compounds for which efflux mechanisms, globally referred to as 'drug efflux pumps,' are numerous and fairly active [1]. Thus, more and more membrane-spanning proteins involved in the outward transport of a surprisingly large variety of drugs have been recognized and characterized over the last years in almost all cell types, from procaryotes and archebacteria through fungi and higher eucaryotes. This now raises the question of which drug is *not* transported. For eucaryotic cells, drug efflux pumps have been viewed by many authors as complementing the cytochrome P_{450}—or other enzyme-based detoxification systems (e.g. Ref. 2) to achieve efficient protection against 'chemical invaders'. Both systems, indeed, show broad specificity and may even work in synergy (see, for example, the concept of a concerted barrier in enterocytes [3, 4]). Drug efflux, indeed, decreases the load on enzyme-mediated detoxification systems, thereby avoiding their saturation, while chemical modifications by the enzyme-based systems, which usually increase the amphiphilicity of drugs, provide drug pumps with better substrates [2, 5]. Moreover, most drug efflux pumps have a broad substrate specificity and, therefore, may deal with a wide range of drugs of completely unrelated pharmacological classes. The present commentary will focus on antibi-

† Corresponding author: F. Van Bambeke, Pharm.D., UCL 73.70 avenue E. Mounier 73, B-1200 Bruxelles, Belgium. Tel. (32) 2-764-73-78; FAX (32) 2-764-73-73; E-mail: vanbambeke@facm.ucl.ac.be

§ Present affiliation: DG Research, European Commission, Brussels, Belgium.

otics, since the role of drug efflux mechanisms, as a major cause of bacterial resistance, has been recognized only recently. It therefore needs to be stressed and examined in detail if one wishes to cope with this major challenge in anti-infective therapy. Quite interestingly, also, it now appears that antibiotic transport mechanisms play important roles in eucaryotic cells by modulating the pharmacological and toxicological profile of antibiotics. Thus, the identification and characterization of the corresponding genes and gene products, in bacteria as well as in eucaryotic cells, have not only an immediate, fundamental interest but also open interesting perspectives for new and more rational developments in chemotherapy.

STRUCTURE AND PHYLOGENY

Transporters can be classified on the basis of three main criteria, namely the energy source, the phylogenic relationship, and the substrate specificity. A 4-digit nomenclature has been proposed recently, constructed in analogy with enzyme nomenclature and in which the first group of digits refers to the mode of transport and energy source, the second and the third to the phylogeny (superfamilies and families), and the fourth to the substrate ([6, 7]; see also the web site <http://www.biology.ucsd.edu/~msaier/transport/titlepage.html>). The so-called **primary active transporters** use various forms of energy and constitute the bulk of the drug efflux pumps in eucaryotic cells ([7]; drug efflux transporters are classically energized by ATP). The **secondary active transporters**, acting as symports and antiports (i.e. coupling the drug efflux to the downhill transport of an ion along a concentration gradient), are predominant in bacteria [6]. Within each of these two main classes, phylogenic studies have led to the recognition of superfamilies, families, and clusters, in correlation with their substrate specificity [6]. Yet, most drug efflux pumps confer a multidrug resistance phenotype, corresponding to the large variety of substrates they may recognize, including several classes of antibiotics as well as non-antibiotic drugs. Antibiotic-specific efflux pumps appear to be restricted to those organisms producing antibiotics and are often an integral part of the corresponding biosynthetic pathway [8].

Table 1 lists the main drug efflux pumps acting on antibiotics described thus far, within the corresponding families of drug transporters. We also mention in which organisms they are mainly found (common bacteria, the special category of antibiotic-producing organisms, common fungi, and mammalian cells). The secondary active transporters, being of a simpler structure, will be presented first. In this group, pumps acting on antibiotics are found in the so-called SMR* family, the MET family, the RND family, the MFS, and the MAR family. SMR have been found thus far only in bacteria [9], whereas MET [10, 11] seem to be restricted to superior eucaryotes. Other families are widely distributed, but antibiotic efflux pumps have not been described in all organisms (viz. the RND [12]). The SMR family can be divided into two groups of gene products; one of them is immediately related to drug efflux, whereas the other is not, which suggests that evolution from the ancestor transporter towards a gene product with a drug efflux phenotype occurred only once [9, 13]. Highly conserved motifs are involved in transport activities as well as in binding of the substrates. MET members present a general organization similar to that of SMR members but are characterized by signal motifs rich in tyrosines at their C-termini, which direct them to intracellular compartments of eucaryotic cells [11]. Members of the RND superfamily share common topological features, namely 2 large extracytoplasmic loops and 12 transmembrane segments resulting from an internal duplication of a gene encoding a 6-transmembrane segment (see Ref. 12 for review). All the known members of this superfamily have the function of efflux transporter, but proteins conferring multidrug resistance are grouped in two of the seven families recognized in this superfamily. Thus, the HAE1 family is largely predominant and includes the well known drug efflux pumps of Gram-negative bacteria with very broad substrate specificity. They probably all derive from a single ancestor [13, 14]. In contrast, there is only one member characterized so far in the HAE2 family. Yet, *Mycobacterium tuberculosis* possesses 10 genes encoding proteins of this family, which could explain the poor sensitivity of this organism to many common antibiotics, if all of them were to correspond to antibiotic efflux pumps [12]. The situation is more complex for drug efflux pumps belonging to the MFS [15, 16]. Indeed, drug-specific and multidrug efflux pumps appear to be randomly interspersed in families (see, for example, the DHA2 [also called DHA14] family), indicating that narrowing and broadening of specificity have occurred repeatedly during evolution [13, 14]. Yet, they are also thought to derive from a common ancestor. Moreover, sequence analysis suggests that a simple duplication of a gene encoding a 6-transmembrane segment protein led to the appearance of the 12-transmembrane segment family (DHA1 [also called DHA12]). Then, the 14-transmembrane segment family (DHA2 [also called DHA14]) evolved from the insertion of an increasingly hydrophobic central loop of the DHA12 precursor into the membrane (the DHA12 family actually displays a long intracytosolic peptide loop running between the 6th and the 7th transmembrane segments, which may have provided the necessary scaffold for these two additional membrane spanning segments seen in DHA14).

* *Abbreviations*: ABC, ATP Binding Cassette; CFTR, Cystic Fibrosis Transmembrane conductance Regulator; CT, Conjugate Transporters; DHA, Drug:H^+ Antiporters; HAE, Hydrophobic Amphiphilic Efflux; MAR, Multi Antimicrobial Resistance; MET, Multidrug Endosomal Transporters; MDR, Multi Drug Resistance; MFS, Major Facilitator Superfamily; (c)MOAT, (canalicular) Multispecific Organic Anion Transporters; MRP, Multidrug Resistance Proteins; OAT, Organic Anion Transporters; OCT, Organic Cation Transporters; PgP, P-glycoprotein; PDR, Pleiotropic Drug Resistance; RND, Resistance Nodulation Division; SET, Sugar Efflux Transporters; and SMR, Small Multidrug Resistance.

Some of the conserved motifs throughout the MFS have been shown to play a role in activity. The MAR family presents no sequence homology with MFS, but a similar topological organization [17].

Within the group of primary active transporters, the ABC drug efflux pumps have been classified extensively in families according to structural homology [18–22]. More recently, they have been distinguished phylogenetically on the basis of their import or export activity [23]. Whereas import pumps are present only in procaryotes, efflux pumps are maintained in both procaryotes and eucaryotes, suggesting that selection of the transport directionality occurred before divergence between procaryotes and eucaryotes [23]. ABC domains generally present a high degree of homology, whereas transmembrane domains differ between transporters, and might contribute to defining their substrate specificity [18]. Considering efflux pumps only, two families, the MDR and the CT2, deserve special attention since they correspond to the most studied and pharmacologically important transporters. They also show functional interchangeability between different types of organisms [24, 25]. The MDR family, which includes the well-known P-glycoprotein in eucaryotes (PgP, a product of the *MDR1* gene as shown in Table 1), is responsible for the efflux of a wide range of drugs besides antibiotics, including anticancer agents. The CT2 family, comprising MRP in superior eucaryotes and Yor1 in *Saccharomyces cerevisiae*, also is involved in the efflux of many drugs, again including antibiotics and anticancer agents [2, 21, 26–28]. This family is phylogenetically close to the CFTR family, a chloride channel, which, however, does not transport drugs (its mutation causes cystic fibrosis). The PDR transporters share several biochemical features with the human PgP [19–21] and constitute the major class of ABC drug efflux pumps in yeasts and fungi. Finally, proteins from the DrugE1 family are involved in drug-specific efflux in antibiotic-producing organisms [13, 23].

ORGANIZATION AND FUNCTION

Figure 1 shows in a combined fashion the topological organization of the main antibiotic-extruding pumps presented in Table 1 together with a schematic view of their mode of operation and the type of antibiotics transported. The mechanisms of transport and of substrate recognition, however, remain largely unknown in most instances, and many of the current views are based on extrapolations from data obtained with transporters of physiological substrates (the assumption is that proteins deriving from a common ancestor have maintained sufficient similarity not only of structure but also of function throughout evolution). SMR, RND, and most MFS transporters (and probably also the MET and MATE transporters) use a proton gradient as the driving force. A minimum of 12 transmembrane segments seems required for activity, so that SMR transporters are probably organized in trimers [29, 30]. The putative mechanism of drug transport, as established by site-directed mutagenesis of a SMR transporter, could involve the following steps: (i) exchange between the drug and a proton fixed on a charged residue; (ii) translocation of the drug by a series of conformational changes driving it through a hydrophobic pathway; and (iii) replacement of the drug by a proton in the external medium and return to the initial conformational state [14, 31]. The overall result of the transport is therefore an exchange between the drug and a proton (antiport). The residue responsible for proton exchange in SMR could be a conserved glutamate [32]. The same mechanism probably applies to MFS transport, but the proton exchanger may be a conserved arginine [29] (again, a conserved acidic residue may be involved in the recognition of positively charged substrates [33]). This mode of extrusion explains why both SMR and MFS transporters preferentially extrude cationic molecules (see below). Less is known about transport by RND members. Like SMR and MFS transporters, however, RND transporters possess highly conserved charged residues in their transmembrane segments, which may play an important role in substrate binding or proton transport [34]. In Gram-negative bacteria, the 2 large extracellular loops of RND are thought to interact with two other proteins [34], namely the 'membrane fusion protein' (connecting the inner membrane to the outer membrane) and the 'outer membrane protein' (crossing this outer membrane). This tripartite protein complex allows the bacteria to extrude the substrate directly into the external medium, bypassing the periplasm and thereby amplifying the efficacy of the transporter. It is now proposed to be a common feature for RND, MFS, and ABC efflux pumps in Gram-negative bacteria, with the 'membrane fusion proteins' differing between transport systems, whereas the 'outer membrane proteins' are probably common to all three transporters [35]. ABC transporters, which contain two ATP binding cassettes (ABC domains), derive their energy from the hydrolysis of ATP. An additional characteristic of MRP transporters is that their activity is strictly dependent on the presence of glutathione. Whether glutathione directly activates the transporter, or is itself co-transported with the drug, or even forms a conjugate to the drug, is not clear, but its weak ionic character is thought to be critical (see Ref. 26 for review). As for proton antiporters, a conformational change of the ABC protein is necessary for drug extrusion and probably is triggered by drug binding and ATP hydrolysis [36, 37].

The exact mechanism of drug transport is still controversial. Among the different models that have been proposed, the two most likely ones present efflux pumps as acting either like hydrophobic 'vacuum cleaners' or like flippases. In the first model, the drug is thought to move freely into the lipid phase of the membrane, then reaching the protein and its central channel, from where it is actively expelled outwardly. In the second model, the drug is also thought to reach the protein from within the membrane, but then would be flipped to the outer layer (as proposed for phosphatidylcholine flip-flop under the action of flippases).

TABLE 1. Classification* and illustrative examples of drug efflux pumps extruding the antibiotics used in clinical practice†

Mechanism of transport	Superfamily‡	Family‡	Bacteria	Antibiotic producers	Fungi	Mammals
#2.(1–77): Secondary active transporter (symports, antiports, uniports)	#2.7.(1–2) SMR Small Multidrug Resistance	#2.7.1. Small Multidrug Efflux	• EmrE of *E. coli* (ery, sulf, tet) Mmr of *M. tuberculosis* (ery)			
	#2.74.(1) MET Multidrug Endosomal Transporter	#2.74.1.				• MTP of *Mus musculus* (ery)
	#2.6.(1–7) RND Resistance Nodulation Division	#2.6.2. HAE1 Hydrophobe Amphiphile Efflux	• Acr of *E. coli* (βlac, chl, ery, fus, nal, rif, tet) • Mex of *P. aeruginosa* (ag, βlac, inhib, βlac'ase, chl, fq, fus, 14 mL, 15 mL, rif, tet) • MtrD of *N. gonorrheae* (βlac, chl, ery, fus, rif, tet) • AmrB of *B. pseudomalei* (ag, ery)			
		#2.65. HAE2 Hydrophobe Amphiphile Efflux		• Actll3 of *S. coelicolor* (actinorhodin)		
	#2.1.(1–29) MFS Major Facilitator Superfamily	#2.1.2. DHA1 Drug: H^+ Antiporters-1 12 spanners (DHA12)	• TetA, B, E of *E. coli* (chl, nal, tet) • TetC of *P. aeruginosa* (tet) • TetH of *P. multocida* (tet) • CmlA of *P. aeruginosa* (chl) • Bcr of *E. coli* (sulf) • NorA of *S. aureus* (chl, fq, tet) • Blt of *B. subtilis* (chl, fq)		• CaMDR1 of *C. albicans* (azo, chl) • Flr1 of *S. cerevisiae* (azo)	• VMAT1 of *Rattus norvegicus* (monoamines)
		#2.1.3. DHA2 Drug: H^+ Antiporters-2 14 spanners (DHA14)	• EmrB of *E. coli* (nal) • MdfA of *E. coli* (ag, chl, ery, fq, rif, tet) • LfrA of *M. smegmatis* (fq) • TetK of *S. aureus* (tet)	• Ptr of *S. pristinaespiralis* (pris, ref) • LmrA of *S. lincolnensis* (lin) • Pur8 of *S. lipmanii* (pur) • RifP of *A. mediterranei* (rif)	• Atr1 of *S. cerevisiae* (azo)	
		#2.1.19 OCT Organic Cation Transporters				• Oct1 of *R. norvegicus* (organic cations, xenobiotics) [human homologs: Oct1–2] • Oat1 of *R. norvegicus* (organic anions, βlac) [human homologs: Oat1–3]

		#2.1.20. SET Sugar Efflux Transporters	• SetA of *E. coli* (sugars; ag)			
		#2.1.21. DHA3 Drug: H^+ Antiporters-3 12 spanners	• MefA of *S. pyogenes* (14ml, 15ml, ole) • MefE of *S. pneumoniae* (14ml, 15ml) • Cmr of *C. glutamicum* (chl, ery, pur, tet) • TetV of *M. segmatis* (tet) • Tap of *M. fortuitum* and *M. tuberculosis* (tet)			
	#2.66.(1) MAR Multi Antimicrobial Resistance	#2.66.1.	• NorM of *V. parahaemolyticus* (ag, fq)			
#3.(1–11): Primary active transporters	#3.1.(1–70): ABC ATP Binding Cassette	#3.1.35. DrugE1 Drug Exporter-1	• MsrA of *S. epidermidis* (ery)	• OleC of *S. antibioticus* (ole) • SrmB of *S. ambofaciens* (ml) • Tirc of *S. fradiae* (tyl)		
		#3.1.47. DrugE2 Drug Exporter-2	• LmrA of *L. lactis* (drugs)			
		#3.1.61. MDR Multidrug Resistance				• MDR1 of *H. sapiens*[§] (phospholipids; fq, lm, ml, rif, tet)
		#3.1.65 PDR Pleiotropic Drug Resistance			• Pdr5 of *S. cerevisiae* (azo, chl, ery, lm, tet) • Snq2 of *S. cerevisiae* (azo) • CDR1 of *C. albicans* (azo, chl) • AtrA, B of *A. nidulans* (ag, azo)	
		#3.1.67 CT1 Conjugate Transporter-1			• Ycf1 of *S. cerevisiae* (conjugates)	
		#3.1.68 CT2 Conjugate Transporter-2			• Yor 1 of *S. cerevisiae* (ery, tet)	• MRP1–6 of *H. sapiens*[‖] (conjugates, phospholipids; fq)

*This classification, based on that proposed in Refs, 6 and 7, considers first the mode of transport and energy source, and within each mechanism ranks the transporters phylogenetically. The first digit in each number refers to the type of transporter, the second one, to the (super)family and the third one, to the (sub)family. Intervals given between brackets correspond to the number of families identified so far in a superfamily, or of subfamilies in a family. Note that mechanism #1 and #4 and the corresponding transporters are not shown here since they do not correspond to drug efflux pumps. In each family, we present only those subfamilies where antibiotic transporters have been evidenced. This classification is updated regularly. (see http://www-biolog.ucsd.edu/~msaier/transport/ titlepage.html; http://www-biolog.ucsd.edu/~msaier/transport/2_1.html; /2.6.html; /2−7.html; /2−66.html; /2_74.html; /3_1.html).

†Illustrative examples of known antibiotic transporters; substrate lists are not exhaustive but correspond to the present state of knowledge (only the main clinically relevant antibiotics and antifungals transported are shown; for some transporters, we give the physiological substrate if known). Abbreviations: ag, aminoglycosides; azo, azoles; βlac; β-lactams; inhib βlac'ase, inhibitor of β-lactamase; chl, chloramphenicol; ery, erythromycin; fq, fluoroquinolones; fus, fusidic acid; lm, lincosamides; ml, macrolides (14ml, macrolides with a 14-atom macrocycle; 15ml, macrolides with a 15-atom macrocycle); nal, nalidixic acid; ole, oleandomycin; pris, pristinamycin; pur, puromycin; rif, rifampicin; sulf, sulfamides; tet, teracyclines; and tyl, tylosine.

‡Some superfamilies and families are sometimes referred to as families and subfamilies when the number of members in the group is small.

§MDR1 is also referred to as PgP.

‖ MRP2, MRP3, MRP4, and MRP5 are also referred to as cMOAT, MOAT-D, MOAT-B, and MOAT-C [116].

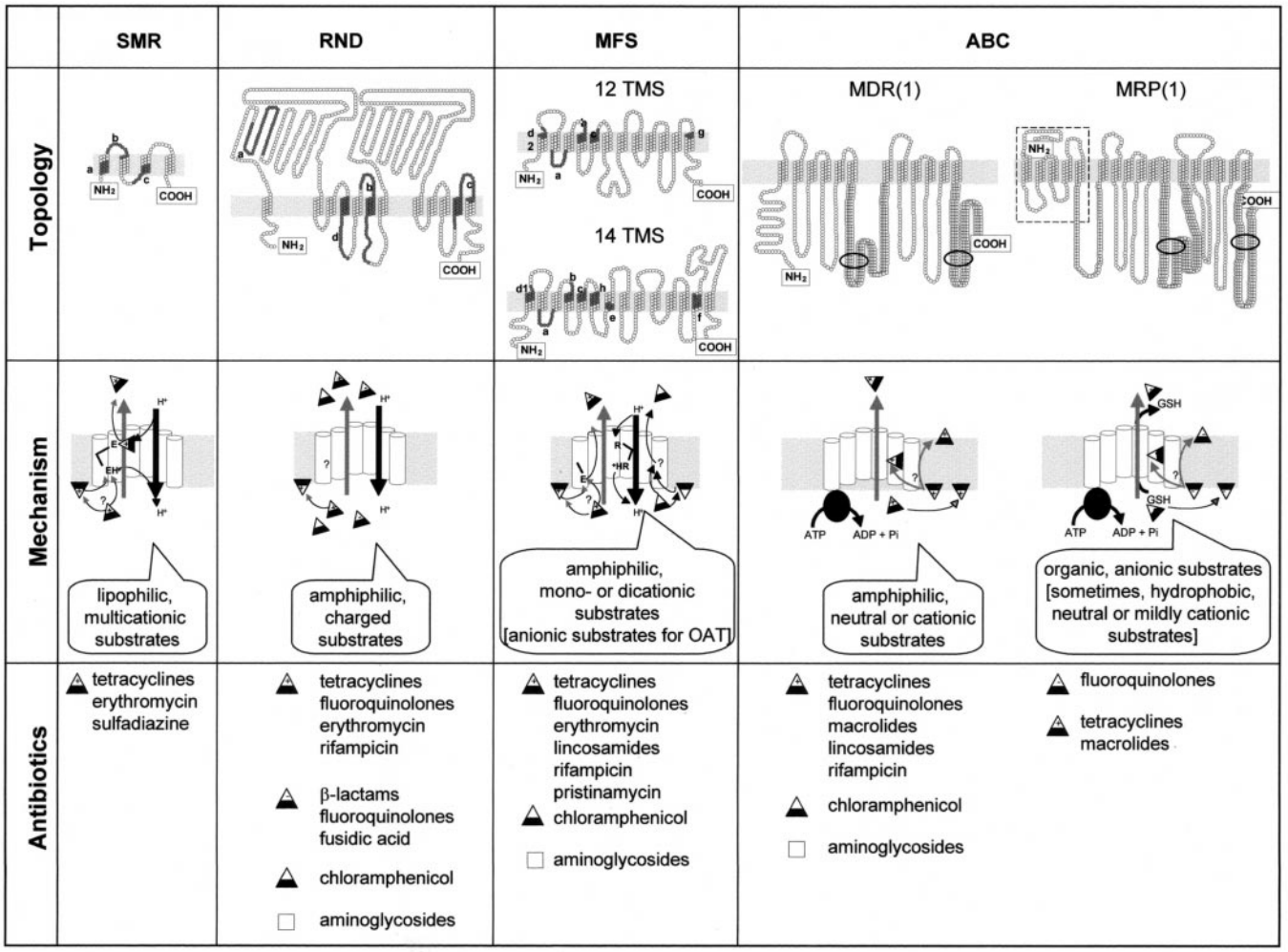

FIG. 1. Topology, mechanism of action, and typical substrates of the main classes of antibiotic efflux pumps (constructed from data and schemes adapted from Refs. 14, 117, and 118). The MET and MAR families are not represented since information on those pumps is still scanty (MET pumps possess 4 transmembrane segments, like SMR pumps; MAR pumps present the same topological organization as MFS). *Topology*: the membrane is represented in grey, with the extracellular milieu at the top of each scheme, and proteins as a chain of circles, the solid ones corresponding to conserved motifs (identified by letters). In ABC transporters, the locations of the ATP binding cassettes are indicated by two black circles. The 5 transmembrane segments at the N-terminal part of MRP (in the discontinuous line square) are present in MRP1, MRP2 (also called cMOAT), and MRP3 [116]. Numerous other organizations of ABC and transmembrane domains have been proposed (for instance, a mirror image of that shown here for the Pgp, or ABC transporters in which transmembrane segments and ATP binding domains are not fused [22]). *Mechanism of action*: SMR, RND, and MFS transporters are drug-H^+ antiporters. H^+ probably is exchanged from a conserved glutamate (E) of the 'a' conserved domain in SMR and from a conserved arginine (R) of the 'b' conserved domain in MFS. Recognition of cationic drugs may imply the same conserved glutamate for SMR transporters and a conserved acid residue (glutamate or aspartate; E in the figure) in the 'd' domain for MFS transporters. No corresponding data are available so far for RND transporters. ABC transporters involved in drug efflux use ATP as an energy source. In all types of transporters, the drug seems to be extracted from the membrane rather than from the cytosol. The transporter then could act as a flippase (catalyzing the flip-flop of the drug from the inner to the outer face of the membrane) or as a 'vacuum cleaner' (moving the drug from the membrane to the central domain of a channel closed to the cytosolic face of the membrane but open to its extracellular face, as already shown for MDR [also known as PgP]). MRP transporters also require the presence of glutathione, which could be conjugated to the drug prior to or during extrusion. *Antibiotics*: classes of antibacterial agents for which transport has been described for at least one pump in each family are grouped according to their general physicochemical character (see Fig. 2).

In contrast to the previous, more conventional models of simple pore-like channels oriented towards export, which pick up their substrate from the cytosol and orient it towards the outside of the cell thanks to a regulator valve, the two models presented assume that the drug is extracted primarily from the inner phase of the membrane. Indeed, strong membrane anchoring is probably a common requirement for substrates of drug efflux pumps. Moreover, transport has been suggested to occur not only from the cytosolic face of the membrane of eucaryotes and Gram-positive organisms (see data with the MFS transporter QacA of *Staphylococcus aureus*; [38]), but also from both the inner and the outer leaflets of the inner membrane of Gram-negative organisms, ensuring clearance from the cytosol as

well as from the periplasm (see the model of extrusion by RND transporters in Refs. 39 and 40). Several lines of experimental evidence, which, however, are based most often on studies with non-antibiotic substrates, support this assumption. First, the only common feature of all pump substrates is a liposolubility that must be sufficient to ensure a proper penetration into the bilayer [41]. Second, substrate or inhibitor molecules can compete with lipophilic fluorophores for insertion into the membrane [41, 42]. Third, lipophilic fluorophores are themselves substrates of the pumps [43]. Fourth, the rates and kinetics of efflux are not directly related to the cytosolic drug content [41, 44]. The structural characteristics of MDR proteins rather favor the hypothesis of a vacuum cleaner. Indeed, the 12 transmembrane domains are organized in such a way that they form a large aqueous pore, open to the extracellular medium but closed towards the cytosol (somewhat like an empty, open bottle or beaker floating on the surface of water [44, 45]). In addition, these transmembrane segments are rich in aromatic amino acids, which could help the hydrophobic substrates to travel into this channel [46]. Functional studies, in contrast, favor the flip-flop hypothesis, since the physiological transporters of phospholipids and of glutathione conjugates, which belong to the MDR and CT2 families, respectively, are known as flippases [47–49].

ANTIBIOTIC SUBSTRATE SPECIFICITY

A key characteristic of the antibiotic efflux pumps is the variety of molecules they may transport, which actually can be related directly to their well-known poor substrate specificity. Considering the pharmacochemical aspects first, it is clear that only very minimal common structural determinants are necessary to obtain detectable transport. Nevertheless, for each class of transporters, investigators have tried to determine which substrate features are the most specific. Results available thus far are presented in a summarized fashion in Fig. 1. Through the use of simultaneous multiple disruption of several transporter genes, however, it has become evident that the substrate specificity is very broad and overlapping across a large array of distinct transporters [28, 50, 51]. The substrate specificity of the MET and the MAR transporters, for instance, has not yet been established with sufficient details and, for the MAR transporters, appears dubious since the NorM transporter representative of this family is claimed to recognize both fluoroquinolones and aminoglycosides, two classes of antibiotics with strikingly different physicochemical properties [17]. Although these substrate specificities may appear difficult to establish, a unifying hypothesis is that most, if not all, transporters recognize molecules with a polar, often slightly charged head associated with a hydrophobic domain (see Fig. 2). Unfortunately, the importance of lipophilia is often difficult to ascertain in the absence of published studies with homogenous series of drug derivatives. It, nevertheless, appears striking for the RND multidrug efflux pumps when considering the data available on the β-lactams, for which a transport ranking of cloxacillin > nafcillin > penicillin G > carbenicillin > penicillin N (the latter being almost not transported) has been demonstrated clearly in close relationship with their corresponding octanol/water partition coefficients [52]. Generally speaking, also, the physicochemical properties of the antibiotics transported by a given class of pumps correspond to those of the non-antibiotic drugs as well as of those of the putative physiological substrates. A major discrepancy, however, concerns chloramphenicol, which is extruded by MFS despite its neutral character [e.g. Ref. 53]. Site-directed mutagenesis studies, however, suggest that the recognition of this drug is probably mediated by interactions different from those observed for other antibiotics [33]. Also remarkable is the rarity of pumps for aminoglycosides [54, 55], but this can probably be explained by the high hydrophilicity of these antibiotics, which prevents their entry into cells by nonspecific diffusion (aminoglycoside antibiotics largely mimic polyamines, an essential substrate for many types of cells, and use their inward transport system for entering both bacteria [for activity] and specific eucaryotic cells [causing toxicity; see Refs. 56 and 57 for reviews]; aminoglycoside-producing organisms generally tend to protect themselves not by efflux pumps, but by the production of aminoglycoside-inactivating enzymes [58]). Similarly, the absence of efflux pumps acting on glycopeptide antibiotics has been explained, at least in bacteria, by the fact that these bulky, largely hydrophilic drugs act in the outer space of Gram-positive bacteria, and never cross the bacterial membrane (glycopeptides are inactive against Gram-negative bacteria precisely because they cannot cross the outer membrane of these organisms [59]). But the situation has evolved so quickly for the other classes of antibiotics that we cannot exclude the possibility that efflux will eventually be demonstrated for glycopeptides also if appropriate studies are undertaken. Beyond these specific considerations, it must also be emphasized that a given antibiotic may be a substrate for different types of pumps, so that (i) it may be expelled by different organisms for which no common transporter has been identified so far (giving the false impression that the transporter is ubiquitous), and (ii) modulation of the activity of a given transporter may be compensated for by a modulation in the opposite direction of another transporter, with, therefore, no or little change in the cellular accumulation of the drug (giving the false impression that the drug is not transported). Moreover, a given pump may extrude not only different antibiotics within the same class but also different classes of antibiotics [28, 39, 40, 55, 60]. Finally, a single cell may possess a vast and complex arsenal of efflux pumps allowing for the extrusion of a very broad spectrum of drugs (viz. *Escherichia coli* and *S. cerevisiae*, the complete genome sequencing of which has revealed the existence of more than 250 putative transporters monopolizing 10% of the total genetic material [6, 7]).

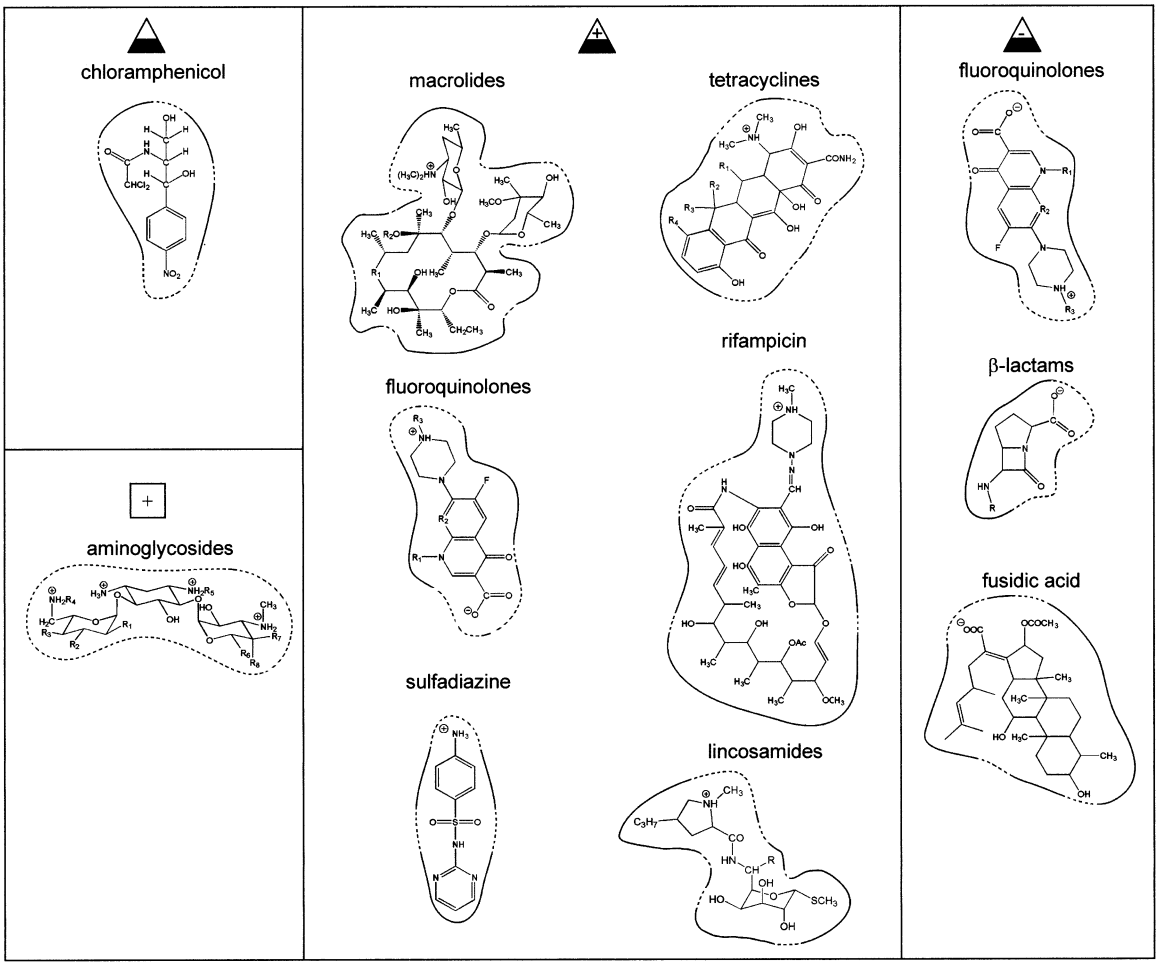

FIG. 2. Structural formulae of the main antibiotics for which efflux has been demonstrated through at least one type of the transporters described in Table 1 and Fig. 1. The molecules are presented so as to show their amphipathic character when appropriate (the zones considered more lipophilic are surrounded by a solid line, and those considered more hydrophilic, by a dotted line). The charged groups are systematically oriented upwards (fluoroquinolones are represented twice, since these can act as cations as well as anions, and accordingly are transported by RND, MFS, MDR, and MRP pumps). The local pH, which may vary widely from one type of organism to another and from the precise location of the pumps, strongly influences the ionization of these groups and their role in recognition by the transporters. The structures shown emphasize the common characters of each class of antibiotics, since structural variations within each class (denoted by the existence of variable substituents [R]) do not appear to alter their recognition properties markedly, systematically, or specifically.

MICROBIOLOGICAL AND THERAPEUTIC SIGNIFICANCE

Considered clinically important only for tetracyclines until a few years ago, antibiotic efflux pumps appear nowadays as a major component of microbial resistance to many classes of major antibiotics [39, 40, 60]. For at least three of them, namely the tetracyclines [61, 62], the macrolides [63], and the fluoroquinolones [64] (which are interesting to analyze in this context since they are totally synthetic, amphiphilic compounds with no known 'natural' counterpart), antibiotic efflux appears sufficient *per se* to confer a medium or high level of resistance, defeating medically applicable treatments of the corresponding infections with these antibiotics. Typical examples include *Streptococcus pyogenes* [65] and to some extent *S. pneumoniae* [65, 66]. Antibiotic efflux may also contribute to the decreased susceptibility of *S. aureus* to fluoroquinolones [64, 67] and of *Pseudomonas aeruginosa* to many classes of antibiotics [68]. Most insidiously, antibiotic efflux may be found in association with other mechanisms, such as antibiotic inactivation, to confer high-level resistance on bacteria. In some respects, this phenomenon bears similarities with the cooperation of drug-extruding pumps and the cytochrome P_{450}-based degradation pathways in enterocytes, which we presented in the introduction of this review. A typical example is given by the cooperation between the penicillin efflux pumps and the β-lactamases, both of which may effectively decrease the concentration of β-lactams in the periplasmic space of Gram-negative bacteria to the point where penicillin-binding proteins are no longer saturated. These bacteria then display the surprising phenotype of high-level resistance without being high-level producers of β-lactamase [69–71]. The situation is more subtle for fluoroquinolones, but illustrates quite well the cooperation between two

apparently unrelated mechanisms of resistance. Resistance to these antibiotics may result from point mutations at the level of the drug targets (DNA gyrase/topoisomerase IV). A single mutation most usually gives rise to only low- or medium-level resistance, and the bacteria may still be considered as sensitive in routine microbiological testing. The combination of two or more mutations, however, will confer high levels of resistance [72, 73]. Because these mutations are easily obtained in many bacterial species *in vitro,* they were considered as being primarily responsible for the resistance seen in the clinic. It now appears, however, that many, if not most, of the organisms with the phenotype of low- to medium-level resistance to fluoroquinolones harbor one or several efflux mechanisms [73–75]. Recent data also show that single point mutations and the existence of fluoroquinolone efflux pumps produce synergistic effects [74, 76]. We speculate that efflux pumps, by decreasing the cellular concentration of fluoroquinolones, may facilitate the selection of mutants with two or more mutations, thereby increasing the risk of emergence of highly resistant organisms. A further striking demonstration of the key role of antibiotic efflux pumps in bacterial resistance is given by the recent observation that the disruption of one or several of their genes, or their direct pharmacological inhibition, results in a major increase of their intrinsic susceptibility to the corresponding antibiotics. It may also decrease the frequency of appearance of resistant mutants [51, 76]. Moving to so-called non-susceptible organisms, it now appears that, in many cases, this phenotype is not due to an intrinsic lack of susceptibility (absence of target or impermeability) as was thought for a long time, but is rather caused by the presence of efflux pumps that are constitutionally very active against the drug under study [40, 54, 76–78]. It is also important to underscore the role of stable mutations at the level of the regulatory genes controlling the expression of multidrug pumps [40]. An example of this multidrug regulation circuit is well described in yeast, where it has been shown that exposure to a given single drug could lead to mutations in regulatory genes provoking the constitutive and simultaneous overexpression of several multidrug efflux pumps (of different types). This results in the irreversible acquisition of a phenotype of multidrug resistance (see Ref. 79 for review), a situation commonly observed in pathogenic fungi resistant to multiple drugs, which indeed often overexpress multidrug efflux pumps [80, 81]. Moving to bacteria, we now know that patients infected by *P. aeruginosa* and treated by a β-lactam alone (or in combination) may become colonized rapidly by strains with a mutation in the regulator of the genes encoding the MexA–MexB–OprM pump [82]. These strains are resistant not only to β-lactams, but also to fluoroquinolones, tetracyclines, chloramphenicol, and trimethoprim. Each drug, presumably, can be expected to also be the inducer of regulatory mechanisms responding to cytotoxic insult by overexpression of drug efflux pumps. Therefore, it is likely that ecological pressure through an inappropriate use of antibiotics will sooner or later lead to the selection of strains showing stable resistance to a wide range of unrelated drugs (this may imply not only bacteria but also other organisms that have been exposed incidentally to the same type of drug). A second, but so far unproven, risk is related to the apparent plasticity and promiscuity of the drug transporters with respect to their potential substrates. This could lead to the selection of transporters with increased efficacy. This self-adaptation of bacteria has been well documented with β-lactamases and aminoglycoside-inactivating enzymes. Each introduction of molecules designed to resist the action of these enzymes has been followed rapidly by the emergence of apparently new enzymes acting against these 'new' antibiotics. Yet, the genetic analysis showed that the new enzymes sometimes differed from the old ones only by single amino acid substitutions [56]. Likewise, in the case of drug efflux pumps, it is known that single amino acid substitutions may affect substrate specificity drastically [6, 33].

The clinical impact of antibiotic efflux pumps on resistance in clinics remains difficult to establish, since we lack large-scale and international statistics comparing their prevalence with that of the other resistance mechanisms. Yet, very recent surveys already published point to alarming figures of 40–90% of some bacterial pathogens (*S. pneumoniae*, *S. pyogenes*, and *P. aeruginosa*) bearing efflux mechanisms for the major classes of clinically available antibiotics ([66, 78, 83–85]; see also abstracts 1211, 1212, 1216–1218, 1220–1223, 1225, and 1228 of the 39th Interscience Conference on Antimicrobial Agents and Chemotherapy [ICAAC], San Francisco, CA, 1999). The abundance of research papers describing antibiotic efflux mechanisms contrasts, however, with the rarity of data from clinical microbiology laboratories. This raises the question of the adequacy of the routine procedures to detect these strains, which most often may be classified as moderately resistant and erroneously assigned to a conventional mechanism of resistance in the absence of further detailed investigation. Multiresistant organisms will need to be screened critically in this context.

Moving now to eucaryotes, it becomes very clear nowadays that the transepithelial movement of several drugs, including antibiotics, implies transport systems. The concerted action of different pumps located both on the basolateral and the apical membranes of epithelial cells has been proposed to account for the preferential transfer of certain antibiotics from the blood to the excretory pathway. This cooperation is best evidenced in the liver, where OAT and cMOAT (also called MRP2, see Table 1) ensure the unidirectional transfer of drugs to the bile (see Ref. 86 for review). It is also suspected to occur in the kidney proximal tubules [87], and secretory transepithelial transport of antibiotics has been demonstrated in the intestine and airway epithelia [88, 89]. Practically speaking, the activity of pumps explains the poor intestinal resorption of several antibiotics [88, 90, 91] and gives a rational explanation for the behavior of the so-called orally available penicillins or cephalosporins. The recognition of the existence of antibi-

otic transporters now may be put into use for more rational design in the future [92]. Similarly, antibiotic transport mechanisms operating in the liver and kidney explain some of the elimination features of β-lactams and fluoroquinolones [93, 94]. The recognition of the existence of antibiotic-extruding pumps in macrophages, which act on β-lactams and fluoroquinolones [95, 96], and, for MDR-expressing cells, on macrolides, tetracyclines, lincosamides, and rifamycins as well [97, 98], has shed new light on the lack of, or potentially reduced activity of, these antibiotics against intracellular bacteria. Antibiotic efflux pumps will, indeed, reduce the amount of drug present in phagocytes to a point where it may no longer exceed the minimal inhibitory concentration at the site of infection. This was clearly demonstrated in the case of *Listeria monocytogenes*, which causes cytosolic infections (addition of an inhibitor of the fluoroquinolone efflux pump markedly increases the activity of these antibiotics [96]). The situation may be more complex for infections affecting the vacuolar apparatus, because efflux pumps in this case could promote the accumulation of antibiotics from the cytosol into these vacuoles, since their membranes partly derive from invaginations of the pericellular membrane [2, 99].

CHEMOTHERAPEUTIC PERSPECTIVES

The search for new anti-infective agents now must take into account the growing problem of resistance. In this context, glycylcyclines and ketolides were designed and/or screened specifically for action against strains displaying the antibiotic efflux pumps recognizing their parent compounds (tetracyclines and macrolides ([100, 101]; see also abstracts 2133, 2137, and 2140 in the 39th ICAAC). The failures encountered with antibiotics designed to specifically resist inactivating enzymes (β-lactamases, aminoglycoside-modifying enzymes, and so forth) show, however, that chemical 'improvements' are likely to be overcome quickly by bacteria. Obtaining specific and potent inhibitors of antibiotic transporters, therefore, appears today as an important objective in anti-infective chemotherapy. Although basic knowledge is still scanty in many areas, several lines of research can be followed safely using either ligand-based or target-based design approaches. Ligand-based approaches have long been the main source of new chemotherapeutic agents and, therefore, could be followed usefully in this case. Yet, they may fall short of an accurate definition of a starting pharmacophore. Indeed, we have seen that there is actually little stringent structural requirement for a drug to be transported. Target-based approaches, on their side, may offer more opportunities for defining a specific ligand, especially since we now have a growing capacity to undertake precise molecular modeling allowing one to define ligands. In this context, it is important to emphasize that effective inhibitors do not necessarily have to be directed against the binding site of the natural substrate. For example, effective inhibitors acting on proteins of the picornavirus capsid have been designed to bind to functional groups situated out of the site of interaction of the protein with its target [102]. Moreover, for members of the ABC transporter superfamily, it also appears possible to inhibit the ATPase activity of the transporter rather than its efflux capacity [44, 103]. Several groups of both academic and industry-based researchers are heavily engaged in the search for pump inhibitors [104–107]. A major difficulty, however, may arise from the fact that antibiotic-extruding pumps could be proteins with important physiological functions, the manipulation of which may cause unexpected toxicities. In this context, efforts directed to specifically inhibit antibiotic-extruding pumps operating only in procaryotes may offer significantly greater chances of effective therapeutic success ([108]; promising compounds have also been presented in this respect at the 39th ICAAC [viz. abstracts 1264–1272]). An indirect therapeutic application of our present knowledge of the antibiotic-extruding pumps could be the use of antibiotic molecules themselves to inhibit the transport of other chemotherapeutic agents such as anticancer drugs [109–113]. The rationale of this approach is that we already possess effective pharmacophores with low levels of toxicity that are backed by long clinical experience [114]. Although this approach may seem attractive at first glance, it will, in our opinion, quickly stumble on the problem of the overuse of antibiotics, which is one of the major causes of worldwide antibacterial resistance. This non-antibiotic use of antibiotics, therefore, may create an uncontrollable problem, not only at the level of the patient but also at that of the community, as clearly exemplified by the use of antibiotics as food additives [115]. We therefore suggest that there is not only room but also a necessity to design truly effective and finely tuned specific inhibitors of antibiotic efflux transporters, which will usefully complement our present anti-infective armamentarium. This could be achieved by concerted genomic and proteomic studies targeted towards the discovery of specific genes and gene products, with complete biochemical and functional characterization in purified systems.

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