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Review Article

RND Efflux Substrates: Antibiotic Resistance Clues

Johan Revol-Tissot, Gérard Boyer and Sandrine Alibert*

Aix Marseille Univ, MCT, U1261 INSERM, SSA, UMR_MD1, France

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Corresponding author:
Sandrine Alibert,
Aix Marseille Univ, Faculté de Pharmacie, 27 Bd Jean Moulin, 13005 Marseille, France,
Tel: +33(0) 491 835 538;
Email: sandrine.alibert@univ-amu.fr

ABSTRACT

Antibiotic resistance could be one of the leading causes of global death rate if antimicrobial development and therapy schemes are not improved. In this context, it is crucial to determine which molecular determinants are responsible for a decrease in the constitutive activity of the antibiotic. RND efflux pumps constitute the first line of defense mediating Gram-negative bacteria. This mechanism is not subject to automatic diagnosis in clinical routine management by antibacterial susceptibility tests, even though it could also limit other resistance mechanisms that can be observed. Reviewing and assessing every antibiotic family and non-active dye compounds MIC data from literature, based on efflux resistant *Escherichia coli* strains combined with physicochemical properties, the objective of this study is to highlight which substituents are responsible for a relatively high or low uptake by AcrAB-TolC and how they affect pharmacodynamic response. Therefore, these data on *E. coli* provide a first study focused on efflux which is less reliant on MIC differences between over-producing and non-overproducing efflux pump strains usually not linked to specific target activity and the impact of efflux pump uptake.

INTRODUCTION

When antibacterials started to be used, including antibiotics and biocides, to fight against bacterial infectious diseases, it heralded the beginning of a new era of modern medicine where new effective tools were likely to tackle bacterial infections that are still rampant in the world today. Penicillin was the first antibiotic found thanks to Alexander Fleming's work in 1928 and its use became widespread in 1945. Unfortunately, this new therapeutic arsenal has given rise to the reality of drug resistance. The 2014 WHO report underlined disturbing epidemiological evidence about acquired drug resistance of many tenacious bacterial pathogens in hospitals and communities, then the Review on Antimicrobial Resistance (AMR) confirmed that AMR could kill 10 million people a year by 2050 and finally a study on the global burden of bacterial AMR was published in 2019 [1] all emphasize a growing concern about one of the leading causes of death in the world. It is particularly worrying since the 2050 estimate figures for global deaths have been reached within 5 years almost achieving a 50 per cent increase. These phenomena contribute to severe loss of antimicrobial activity for compounds that are still expected to be effective. Bacteria, like any living organism has sought to adapt to its environment to survive and evolve through time and space [2]. It has led to selective pressure and to the appearance of antibiotic-resistant mutant in clinical issues [3,4].

The evolution of antibiotic resistance in common bacterial strains provides resistance to a broad range of antibiotics and contributes to Multi-Drug Resistance (MDR) phenotype especially in Gram negative bacteria [5]. It is the case for strains with acquired and intrinsic resistance mechanisms. They are categorized in 2 main types, depending on the nature of the mechanism: the first one is enzymatic degradation, particularly β-lactams, aminoglycosides and some macrolides by hydrolysis or biotransformation [6]; the second one is, intracellular target alterations in bacterial DNA and mRNA, particularly for penicillin and quinolone derivatives in Gram-positive and Gram-negative bacteria [7]. If the two of these resistance mechanisms are specifically related to one or two groups of antibiotics based on pharmacodynamic characteristics, they can also be associated with other membrane effects that enable the bacteria to extend the lag period in response during antibiotic exposure. At membrane level, decreased porin expression [8] and, meaningfully, efflux pump overproduction are major contributors to the loss of effective intracellular antibiotic concentrations as they appear at early exposure and involve all antibiotic classes, leading to the MDR phenotype. Consequently, they constitute their primary resistance barrier [9].

Efflux pumps are ubiquitous protein complexes that actively extrude bacteria-damaging substrates such as antibiotics, detergents, antiseptics, toxins from cytoplasm or periplasm into the external environment. These transporters recognize a wide range of physicochemically varied substrates [10]. There are currently 6 families including ABC (ATP-Binding Cassette), SMR (Small Multidrug Resistance), MFS (Major Facilitator Superfamily), MATE (Multidrug And Toxic compound Extrusion), PACE (Proteobacterial Antimicrobial Compound Efflux) and RND (Resistance-Nodulation-Division) transporters [11,12].

The last two, but more specifically RND transporters that are an energy-dependent tripartite system provided by a proton pump which was initially discovered in Tetracycline resistant *Escherichia coli*, were isolated in the same way as P-glycoproteins in mammalian cells [13]. RND pumps are specific for Gram-negative bacteria due to their tripartite structure, arranged from inner to outer membrane [14,15]. Molecular modeling enables an assessment of the activity of new compounds from models that define interesting physicochemical features connected with pharmacological and pharmacokinetic properties of bioactive agents. However, to improve research strategies, and limit efflux resistance outbreaks, it is imperative to understand the molecular determinant of the physicochemical properties involved in substrates uptake. Since the 90s, many efforts were focused on studies on Structure Activity Relationships (SAR) of antibiotics. In most studies the antibiotic activity is determined with Minimum Inhibitory Concentration (MIC). It remains a very valuable tool for diagnostics, as clinicians can select the most appropriate antibiotic against a specific bacteria based on susceptibility tests [16]. However, only a small number of reviews consider the target site. As a result, their interpretation is less significant especially about efflux because of the diversity of membrane transporters, their complex component structure and function particularly for RND pumps. In this case, the evaluation of antibiotic concentration inside cells is crucial.

In this review, the goal is not to be exhaustive, but pharmacochemical data have been selected according to their relevance. They are directly involved in the evolution of the structure of antibiotics compared with efflux resistance to promote an improvement of the use or design of antimicrobial agents, to encourage further research on antibiotics and make the scientific community aware of the progress that can still be made in this field. A better understanding of the molecular characteristics and the underlying mechanisms limiting uptake and intrabacterial accumulation would thus enable the discovery of novel antimicrobials and of the prevention of resistance.

Antibiotics are part of the therapeutic arsenal that revolutionized the clinical treatment of infected patients. While some can be found in nature (e.g., Penicillin made by a fungus *penicillium*, tetracyclines, macrolides and aminoglycosides isolated from actinomycetes [17]), others derived from natural products are semi-synthetic (e.g., Ampicillin and Amikacin) or fully synthetic (e.g., Norfloxacin). They have a specific action on bacteria or protozoa. They are called bactericidal or bacteriostatic depending on their mechanism of action [18,19]. The AcrAB-TolC archetype efflux pump represents a substantial database source regarding the Scientific Literature. We selected publications evaluating a broad range of antibiotics versus Gram-negative efflux resistant bacterial strains and we

matched the effects of AcrAB-TolC on the physicochemical properties of antibiotics. This work can provide a better understanding of RND efflux contribution to antibiotic activity, especially with MIC measures, depending on efflux resistance levels and the chemical structure of antimicrobials.

Table 1: Summary table of predicted or experimentally determined physicochemical properties of antibiotics and dyes included in this review. The bracketed values indicate the minimum and maximum values of the series, and the parenthesis value represents the mean of the data. Predicted properties as HBA/HBD, rotatable bonds, stereocenters and rings count, including aromatic ones, were calculated using ChemAxon software MarvinSketch v.20.8. LogP and pKa data were either obtained using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2022 ACD/Labs) from SciFinder® or experimentally determined as literature mentioned in the text. Indeed, complexity was computed by Cactvs 3.4.8.18 from PubChem (2021.05.07 release).

ATB family	ATB	HBA	HBD	ROT	CMPX	STRC	LogP	pKa	R	AR
Quinolones	Nalidixic acid	[5] (5)	[1] (5)	[2] (5)	378 (378)	[0] (0)	[1.9] (1.9)	[3.45] (3.45)	[2] (2)	[2] (2)
Fluoro-quinolones	Ciprofloxacin, norfloxacin, ofloxacin, sparfloxacin, clinafloxacin, Enrofloxacin, bazufloxacin, gatifloxacin, fleroxacin, moxifloxacin, lomefloxacin, nadifloxacin, pefloxacin, balofloxacin, difloxacin	[7/9] (7.7)	[1/3] (1.7)	[2/5] (3.2)	[545/727] (618.4)	[0/2] (0.7)	[-0.31/2.91] (1.04)	5/6.4 (5.88)	3/5] (3.5)	[1/3] (1.8)
Fluoro-naphthyridines	Tosufloxacin, gemifloxacin, trovafloxacin, enoxacin	[8/10] (9.5)	[2] (2)	[3/5] (3.5)	[521/770] (681)	[0/2] (1)	-0.2/2.3] (0.59)	[5.8/6.3] (6.01)	[3/5] (4)	[2/3] (2.5)
Penicillins	Penicillin G, penicillin V, ampicillin, piperacillin, mezlocillin, oxacillin, cloxacillin, dicloxacillin, azlocillin	[5/9] (6.9)	[2/4] (2.6)	[4/6] (4.7)	[530/1080] (743.8)	[3/4] (3.4)	[-0.83/2.91] (1.45)	[2.3/3.4] (2.7)	3/4] (3.7)	[1/2] (1.6)
Cephalosporins	Cefotaxime, cefuroxime, ceftazidime, cefepime, cefazoline, cefoxitin, ceftriaxone, cefoperazone	[9/13] (11.4)	[2/4] (3)	[6/9] (7.7)	[740/1250] (920.5)	[2/3] (2.1)	[-1.7/0.26] (-0.91)	[2.3/3.6] (2.72)	3/5] (3.7)	[1/2] (1.6)
Carbapenems	Ertapenem, imipenem, meropenem,	[6/9] (7.3)	[3/5] (3.7)	[5/7] (6)	[491/893] (687.7)	[3/6] (5)	-2.95/ -0.78] (-1.66)	2.9/4] (3.4)	[2/4] (3)	[0/1] (0.3)
Cyclins	Tetracycline, tigecycline, minocycline, chlortetracycline, eravacycline,	[9/11] (9.8)	[5/7] (6)	[2/7] (3.8)	[971/1240] (1078.4)	[4/5] (4.4)	-1.3/2.55] (0.1)	[2.8/4.5] (3.7)	4/5] (4.2)	1] (1)
Macrolides	Erythromycin, azithromycin, clarithromycin, oleandomycin, tylosin, josamycin.	[13/18] (14.8)	[3/5] (4.2)	[6/14] (9.2)	[1090/1560] (1260)	16/21] (18.2)	[1.63/4.02] (2.76)	[7.7/13.1] (9.7)	[2/4] (3)	0] (0)
Aminoglycosides	Kanamycin, puromycin, gentamycin, spectinomycin, amikacin, neomycin	[9/19] (13.7)	[4/13] (9)	[2/10] (7)	[478/872] (687.2)	[5/19] (12.7)	[-8.78/-3.7] (-4.92)	[7/10.2] (8.6)	3/4] (3.2)	[0/3] (0.5)
Dyes	Pyronin Y, acriflavine, propidium iodide, ethidium bromide, Nile Red, Hoechst 33342	[2/5] (3.5)	[0/3] (1.2)	[1/7] (3.7)	[419/664] (517)	[0] (0)	[-4.8/3.44] (-0.4)	2.35/10.97] (4.60)	[3/6] (4)	[3-5] (3.8)

ATB: Antibiotic; HBA: Hydrogen Bond Acceptors; HBD: Hydrogen Bond Donnors; ROT: Rotatable Bond Counts; CMPX: Complexity; STRC: Stereocenter Counts; R: Ring Counts; AR: Aromatic ring Counts.

DISCUSSION

Review approach

We analyzed the pharmacological descriptor MIC, as it constitutes an appropriate index of bacterial strain resistance status and antibiotic sensitivity, with several physicochemical properties. Among pharmacochemical descriptors, we used as structural parameters (Table 1) rotatable bond, ring counts, including aromatic ones, stereocenter counts and complexity, as thermodynamic parameters, octanol/water partition coefficient logarithm LogP and as electronic parameters, negative acidity constant logarithm pK_a, Hydrogen Bond Donor (HBD) and Acceptor (HBA), aromatic and non-aromatic ring counts. All the values described were calculated from calculation software mentioned in graph legend. Experimental physicochemical data were obtained for LogP and pK_a. Firstly, LogP values were taken for some (fluoro)-quinolones and fluoro-naphthyridines [20-24], β-lactams and cyclins [22,25-26], macrolides [27,28], aminoglycosides and dyes [22,28-30]. Secondly, with a similar method, pK_a values were even taken from some (fluoro)-quinolones and fluoro-naphthyridines [24,31-37], β-lactams [25,31,37-39], cyclins [24,37,40], macrolides [27,31,41] and aminoglycosides [29,31,37,42].

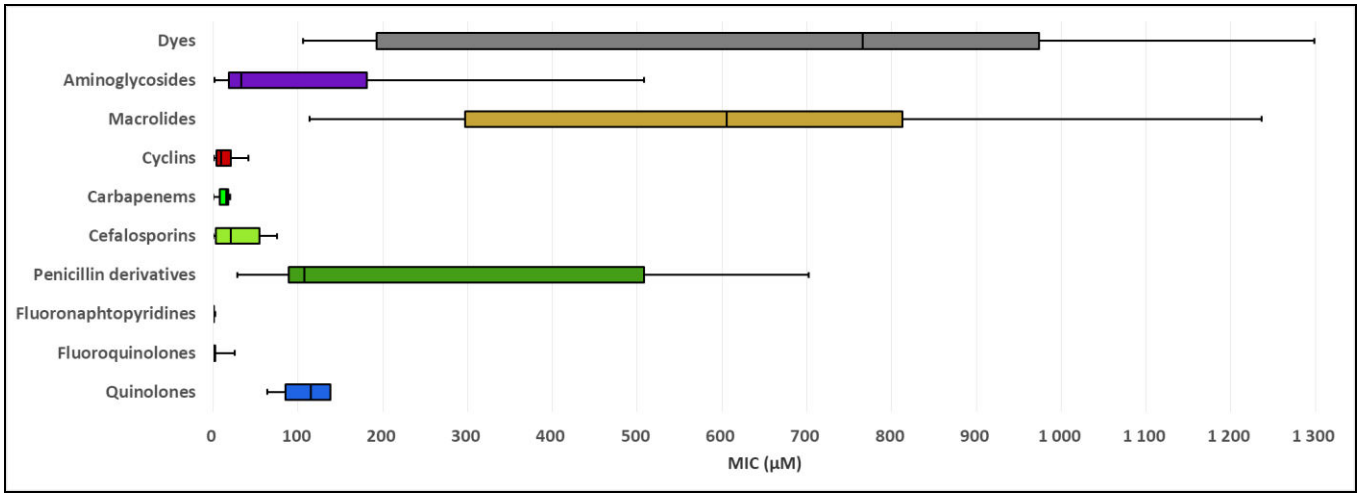

Figure 1A: Box plot representing MIC values according to different antibiotic families and other molecules in efflux-resistant *E. coli* strains.

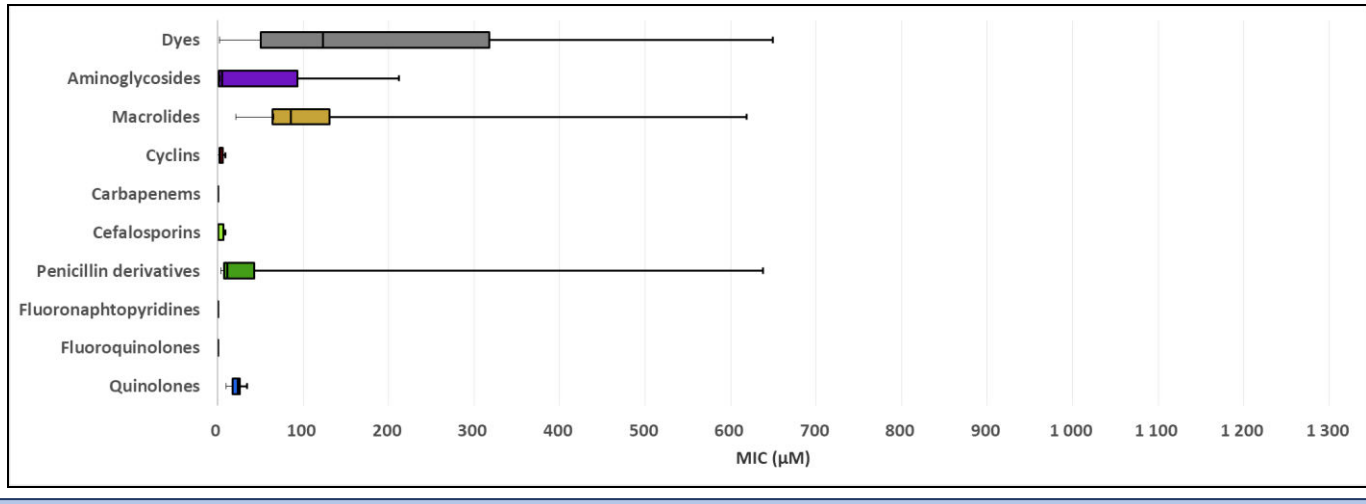

Figure 1B: Box plot representing MIC values according to different antibiotic families and other molecules in wild-type *E. coli* strains.

Since *E. coli* is the most representative AMR pathogen combined with the most widely used antibiotics [1], we studied the following efflux resistant strains from literature MIC data and compared them within a single antibiotic family (Figure 1A): *marR* mutants AG102 (point mutations correlated with loss of *marR* repressor function by inhibition of *marO* promoter binding leading to *marA* overexpression) [8,43-47], AG102B [48,49] and 3-AG100 [50-52], *marA* mutant AG112 and CH164 (*marO* deletion mutation leading to an affinity loss with *marR* repressor and therefore *marA* overexpression) [53,54]. Moreover some recombined strains with plasmids were selected as HN1157/pHSGoxa7 with *acrR* deletion and penicillinase OXA-7 gene plasmid (operon *acrR* and *acrAB* transcription repressor) [55], GC7368/pCLL3431, an AG100A *E. coli* strain with cloned AcrAB gene leading to efflux pump overexpression [56], JM101/pMAQ43, an MDR-derived strain with *ramA* (*acrA* overexpression in absence of *marA* or at least in reduced production) [57] and susceptible AG100A/pET28a-AcrB strain with *acrB* (plasmid containing the gene encoding AcrB leading to AcrAB-TolC overexpression as in GC7368/pCLL3431) [58]. Indeed, we added clinical isolates with AcrAB-TolC overexpression as, for example, G5049 [59,60]. MIC data were collected from these efflux-resistant strains and were compared between antibiotics and other agents to investigate variations in efflux response related to physicochemical properties.

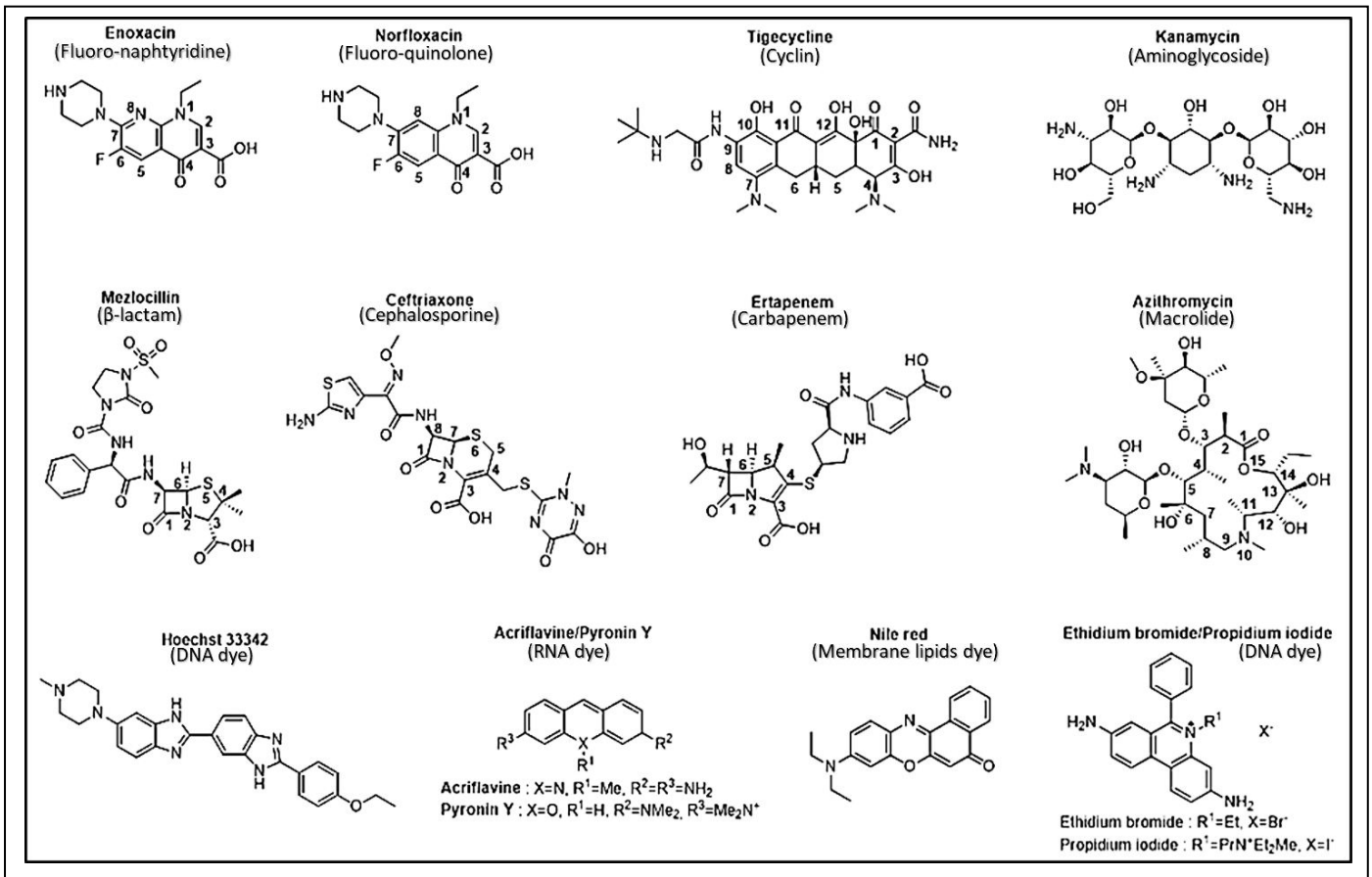

Figure 2: Chemical structures of the most representative antibiotics and dyes from the series included in this review for which the best activity and weakest uptake by AcrAB-TolC efflux pumps are obtained.

Because the average MIC values for different antibiotic families can vary considerably in their efficacy across different experiments, it is necessary to assess whether the observed variations are attributable to the impact of AcrAB-TolC efflux pumps. For this purpose, we also plotted MIC data from wild-type *E. coli* strains (Figure 1B). We selected different wild-type strains as: AG100 [8,43-45,47-48,50-53,56,59], ATCC25922 reference strain from EUCAST 2022 [46,49,54-55,58,60,61] and JM101 [57].

Thus, a SAR study can be defined for this purpose thanks to various structural parameters descriptors mentioned above and used in the corpus of this review. Fluoro-quinolones and fluoro-naphthyridines are the most active antibiotic class on efflux resistant strains with MIC concentration ranging from 0.39 to 2 µM and 0.25 to 0.3 µM, respectively, to be compared to cyclins, carbapenems and cephalosporins. Those three classes share the same MIC concentration range from 3.1 to 50.8 µM. The lowest values are represented by cyclins (8.9 µM), then carbapenems (15.6 µM) and finally cephalosporins (20.9 µM). Next, Aminoglycosides display a narrower concentration range from 16.8 to 266.7 µM which includes quinolones ranging from 85.4 to 137.8 µM. Penicillins have a wider concentration ranging from 88.9 to 400.4 µM, sharing a common feature with other antibiotic classes such as quinolones and aminoglycosides. Finally, macrolides represent the lowest active compounds in the overall *E. coli* series with a MIC range between 297 and 812.9 µM. They have an activity almost twice as low as the less active penicillins. Thus, we find an overall disparity from 600 to 2,400 between the macrolides described as the weakest compounds and the most active ones such as fluoro-quinolones and fluoro-naphthyridines. To improve understanding of the discussed SAR, we depicted all the most representative molecules of each class of drugs in terms of chemical structure (Figure 2).

Quinolone family

Quinolones are the most powerful and widely used antibiotic family nowadays. This class was derived from Nalidixic Acid, the first quinolone discovered by accident as a by-product during the synthesis of Chloroquine [62]. With other quinolones as Piromidic acid and Cinoxacin, they constitute the first-generation quinolones. They are a group of bactericidal compounds that act on DNA replication and mRNA transcription machinery on nuclear sites such as gyrases and topoisomerases, for Gram-positive strains [63]. The interaction of enzyme with molecule results in the formation of a stable complex between the antibiotic-bound enzyme and the DNA strands via covalent bonds [64]. The double strand of DNA is thus broken and, ultimately, DNA replication is stopped, and cell death occurs. Although these precursors lacked significant pharmacological activity against Gram-negative bacteria, they were useful as models for the synthesis of second-generation quinolones or fluoro-quinolones which show more significant activities. Several new generations were synthetized according to their chemical structure such as Ciprofloxacin and Fleroxacin for second generation quinolones or fluoro-quinolones, Levofloxacin and Sparfloxacin to develop third generation antibiotics and lastly Moxifloxacin for the latest [65]. All these compounds exhibit resistance phenomena. Mutations are found at the level of gyrases and topoisomerases which cause a loss of affinity of fluoro-quinolones for their enzymatic target [66]. In Gram-negative bacteria, the efflux phenomenon is a major cause of resistance, especially with the expression of RND-type pumps. These mechanisms, which affect many strains, induce a restriction of their use over time [9]. But some new influx and efflux monitoring methods may help us design new fluoroquinolones as in the case of *E. coli* and may be extended to other *Enterobacteriaceae* and Gram-negative bacteria [8]. In this context, recently, a large set of 6-fluoro-quinolones and 6-fluoro-1,8-naphthyridines has been studied in isogenic K12 strains of *E. coli* expressing different levels of AcrAB pumps. The results on the AG102 strain which overexpresses AcrAB, draw particular attention when comparing the impact of substituents in position N1, C7 and C8 (Figure 2) on the MIC, inversely proportional to the level of efflux pump expression.

First, we can note that there is no difference in activity regarding the structure of the 6-fluoro-quinolone series and the 6-fluoro-1,8-naphthyridines if we compare, for example, Norfloxacin and Enoxacin (Figure 2) which have the same functional groups. The impact of changing the substituents at the C7 and C8 positions is also not significant. On the other hand, an additional effect is observed related to the nature of the chemical function in position N1. Indeed, the MICs are clearly higher for compounds with aliphatic alkyl groups (ethyl of Enoxacin, Norfloxacin, Lomefloxacin, Pefloxacin, or fluoro-

ethyl of Fleroxacin) in this position or they form a tricyclic structure with the C8 position (for Pazufloxacin, Ofloxacin, Nadifloxacin) compared to cyclopropyl or difluoro-phenyl groups of the other molecules of the two series. These results show that the study of the SAR of the N1 chemical functions important for the inhibition of the DNA gyrase are in the same direction as those obtained on a acterial strain overexpressing efflux: difluoro-phenyl>cyclopropyl>ethyl>fluoro-ethyl>methoxy. We can therefore conclude that the N1 substituent is a common pharmacophore for the gyrase and the AcrB efflux pump targets. Alkyl functional groups are hydrophobic. The decisive interaction site reported for AcrB substrates is a hydrophobic trap [67], hence, the strong relationship between the N1 substituent and the efflux pump behavior of molecules. Furthermore, it is established that the AcrB efflux pump are responsible for resistance to structurally unrelated antimicrobial agents [68] because of its broad substrate specificity [69]. Nevertheless, it is known that to increase the selectivity towards a therapeutic target, it is necessary to have a ligand-target complementarity by limiting the molecular degrees of freedom to reach the "active" conformation. This is possible by rigidifying the molecular structure or inserting asymmetric centers. The overall number of rings and the number of aromatic rings tend to be less important for efflux sensitive molecules. Also, the fewer asymmetric centers there are the more molecule sensitivity to efflux increases. Thus, the more selective the molecular therapeutic target is compared to the efflux target, the less effective the polyselectivity of the pump will be.

Structural complexity is thus an important characteristic as it contributes to selective and specific target binding. The complexity index of this family of antibiotics is the lowest. The 1,8-naphthyridine derivatives are the most complex structures of this class of drugs. Except for Enoxacin, the average complexity index of naphthyridines is 734. As seen above, no difference can be found in their scaffolds, so the molecular complexity is in their substituents as evidenced by Nalidixic Acid (naphthyridine with only alkyl groups in N1 and C7 positions) is particularly sensible to efflux. So, increasing scaffold diversity could increase the overall structural diversity of these types of antimicrobial agents. Diversity oriented synthesis could be the best solution to rejuvenate them.

β-lactam Family

β-lactams represent the oldest antibiotic class used to fight against bacterial infections. They include many compound families as penicillins, cephalosporins and carbapenems [70]. These molecules inhibit peptidoglycan synthesis as membrane target, which is a mucopolysaccharide essential component for the bacterial wall integrity regarding pressures due to the external environment [71]. Peptidoglycan layers can be seen within the periplasmic space as a thin line in Gram-negative bacteria and are cross-linked thanks to transpeptidases catalysis called penicillin-binding proteins [72]. These enzymes are found bound to a β-lactam, forming a highly stable penicilloyl-transpeptidase complex through amide function of the β-lactam core that has a very close structure to that of D-alanyl-D-alanine carboxypeptidase, a peptide located on the terminal chain of the peptidoglycan. The enzyme then is blocked and fails to play its catalytic role. This leads to the destabilization of the bacterial wall and thus its degradation [73].

Within this category of antibiotics, several classes have been developed. Penicillins were the primary β-lactam antibiotics used in order to emphasize the enzymatic degradations [74]. Penicillin G and V were initially active against Methicillin Resistant *Staphylococcus Aureus* (MRSA) strains and other Gram-positive bacilli, but new compounds have broadened the spectrum of action by increasing the impact of the antibiotic on certain Gram-negative bacilli by means of synthesis of aminopenicillins (e.g., Amoxicillin). Finally, the development of carboxypenicillins (e.g., Ticarcillin) then marked an increased resistance to β-lactamases and thus entailed a broader spectrum of action including several enteric anaerobes bacteria and *P. aeruginosa* [75]. In this context, the design of β-lactamase inhibitors has led to improved stability of penicillin against enzymatic degradation (e.g., Amoxicillin and Clavulanic Acid) and thus better activity against resistant pathogens [76]. They constitute valuable therapeutic agents since many penicillins are no longer commonly used as monotherapy [77]. For the penicillin class of antibiotics, structural variations are only found in the C7 aminoacyl (Figure 2) side chain of the penam scaffold. Its length is variable with a substituted phenyl group at its end. The first-generation

penicillins have the shortest aminoacyl chain (Penicillins G and V, Ampicillin and Amoxicillin).

In the side chain of Oxacillin derivatives, the oxazole aromatic structure has been inserted between the phenyl and amide functions. These derivatives have the highest MIC. Obviously the higher number of aromatic rings gives them a greater capacity to be substrates of efflux due to AcrB binding site rich in aromatic amino acids. Moreover, all the molecular fragments that constitute it are conjugated, which confers them a coplanarity and thus a weak flexibility characterized by a weak number of rotatable bonds (on average 5). The SAR study shows here that oxazole pharmacomodulation strategy, which improved the absorption of these antibiotics and their resistance to β-lactamase, has increased the efflux resistance compared to Penicillins G and V, and Ampicillin and Amoxicillin. Finally, penams with the lowest level of efflux transport and uptake are those with polyamide chains. But the greater flexibility of penams (up to 6 rotational bonds) is relative, due to the succession of π-∑-*n* conjugate systems that block free rotations around the amide bonds which is reinforced by their cyclisation. A higher amount of HBA and HBD, a greater polarizability and a higher number of asymmetric carbons strengthen the penam molecular complexity which contributes, with lower LogP and pK_a, to reduce sensitivity to efflux while giving a better specificity towards their therapeutic target compared to the hydrophobic AcrB. As mentioned above, increased scaffold diversity allows increased drug potency.

Indeed, the discovery of cephalosporins has broadened the spectrum of activity of antibiotics. The combination of the β-lactam ring with a dihydrothiazine instead of a thiazolidine ring has allowed the insertion of a new substitution position in the structure to increase the molecular diversity. Improvements in their structure have led to the creation of five generations of antibiotics. The first generations (e.g., Cefuroxime) had shown resistance to MRSA and some *Enterobacteriaceae* (*E. coli*, *K. pneumoniae*) and then to β-lactamases [73]. But it was amended from the third generation one (e.g., Cefotaxime, Cefepime) because even if decreased activity against staphylococci and enterococci could be observed, lower β-lactamase affinity and wider spectrum of action to Gram-negative bacteria and MRSA were highlighted [78]. The most recently approved generation of cephalosporins known as Ceftolozane are currently used in combination with β-lactamase inhibitors for high potent activity against enzymes overproducing pseudomonal pathogens [79]. Cefalexin was in fact prepared from the sulfoxide ester of the corresponding penicillin by ring expansion [80]. Thus, in general, cephems have a better antibacterial activity than penams which is confirmed in efflux-resistant *E. coli* strains. Cefazolin and Cefoxitin show structural analogies with penicillins such as Mezlocillin, Piperacillin and Azlocillin, namely the length and flexibility of their side chains and the number of HBA and LogP. The insertion of an O-substituted oxime function has made it possible to lengthen and cross-link the side chain of third and fourth generation cephalosporins, significantly reducing the MIC by increasing the flexibility (8 rotatable bonds) of these antibiotics and the number of HBA (10-13). This results in a higher molecular complexity (740-1250) which strongly participates in decreasing hydrophobicity and therefore sensitivity to efflux, for better inhibition of cell wall biosynthesis and better bactericidal effects.

Carbapenems have similar structural characteristics but also differences that contribute to their broad-spectrum activity, high potency and to be considered as last resort antibiotics. They are derived from penicillin by effect of 3-positions substitutions in thiol groups. The most representative is Imipenem, used in cases of Gram-negative bacilli resistance against third cephalosporin generation by overproduction of enzymes [81]. Resistance to carbapenems can also be observed for MRSA and for the spread of Carbapenem-Resistant *Enterobacteriaceae* generated by the synthesis of β-lactamases specific group called carbapenemases [82]. The latest widely available antibiotic is Doripenem with higher chemically enzyme stability and high potency against Gram-negative bacteria [83]. First, their side chains differ from those found in penams and cephems: a hydroxyl group at the C7 position of the β-lactam ring and an amino functional group at the C4 position of the dihydropyrrole ring starting with a sulfur atom (Figure 2). Hydrogen atoms at the C6 and C7 positions have a *trans* configuration compared to the *cis* of penicillins and cephalosporins. Thus, if the carbapenems complexity index remains in the same order as that of other β-lactamines, the higher average number of asymmetric carbons (up to 6

carbons), the absence of aromatic ring (except one for Ertapenem) and a very low hydrophobicity (on average -1.66 against -0.90 and 1.45 respectively for cephems and penams) give them a very low efflux substrate character. The time during which the intrabacterial antibiotic concentration is higher than MIC is then improved. This allows us to say that these physicochemical and structural properties are the parameters that best correlate with the antimicrobial agent efficacy.

Cyclin, macrolide, aminoglycoside families

Cyclins, macrolides, and aminoglycosides act by inhibiting protein synthesis thanks to ribosome subunit interactions. This is a very important step for protein biosynthesis after mRNA translation [84].

Cyclins: Cyclins are part of a class of antibiotics derived from Tetracycline that can penetrate eukaryotic cells. That is why they are used to target intracellular parasitic bacteria. These compounds are bacteriostatic and act by 30S ribosome subunit inhibition through tRNA-A-site interaction inhibition. Their spectrum of action goes as far as fighting off Gram-negative and Gram-positive bacteria with resistance phenomena from naturally resistant *Enterobacteriaceae*, anaerobic pathogens and *Pseudomonas aeruginosa*. Doxycycline remains the most widely used therapeutic agent and the most recommended, clinically speaking [85]. Mutations in the ribosomal complex, as well as efflux resistance phenomena due to bacteriostatic activity, have unfortunately contributed to the emergence of mutants with RND (*mar* locus mutation) and MFS (*tet* protein) family pumps overexpression. Ribosomal mutations, efflux resistance and ribosomal protection by greater tetracyclines-A-site dissociation constant (K_D) have dramatically decreased the pharmacological activity of this class of antibiotics, for both past and novel derivatives [86,87].

Tetracycline family compounds, with the common octahydro-naphthacene-carboxamide basic structure essential for activity, enolized tricarbonyl-methane and phenolic diketone systems from C1 to C3 positions and from C10 to C12 respectively (Figure 2), have an amphoteric behavior also due to the presence of the dimethylammonium group at C4. The activity variations are particularly ascribed to substitutions at C6, 7 and 9 positions. The most favorable are the substitutions of the aromatic ring by a dimethylamino or fluoro group at C7 and an aminoalkyl chain branched on an acylamino function at C9. As for other families of antibiotics, the presence of a long flexible chain of nitrogenous functions strongly reduces the substrate character of AcrB-type efflux pumps in *E. coli* (by a factor of 5 between the MIC of Minocycline and Tigecycline for example). The decisive physicochemical parameters are the number of HBA and HBD (optimum at 11 and 7 respectively), the number of rotational bonds (optimum at 7), and the complexity index (>1000). In the case of tetracyclines the number of stereocenters, the LogP and the number of rings in particular the aromatic rings are less significant owing to the common nature of the octahydro-naphthacene scaffold known to interact with many different pharmacological targets. Doxorubicin and minocycline were co-crystallized in a putative AcrB binding pocket consisting of phenylalanine residues F136, F178, F610, F615, F617, and F628 [88]. This suggests that the broad substrate spectrum of AcrB is the result of interactions with hydrophobic amino acids and, to a lesser extent, with polar residues in the binding site. Therefore, this antibiotic class would remain limited by AcrAB-TolC uptake even if its basic pharmacophore is improved. Other synthesis strategies must be studied for chemical optimization.

Macrolides: Macrolides are also bacteriostatic compounds that act by 50S ribosome subunit inhibition which is dissociated from tRNA. They have a medium spectrum of action and show broad resistance to *Enterobacteriaceae*, *Pseudomonas* and some Gram-positive bacilli because of methylation on the 23S ribosomal RNA subunit. There are different classes of compounds ranging from 14 atoms (e.g., Erythromycin) to 16 atoms (e.g., Josamycin). Poor activity is observed due to slow accumulation in Gram-negative bacteria because of low permeability without overlooking RND and ABC efflux pump intervention which dramatically decreases effective intracellular concentration of these antibiotics [89,90]. Since the discovery of Erythromycin A, intense efforts have been made to prepare macrolide analogues with enhanced potency and physicochemical properties. Even though the physicochemical and molecular characteristics set macrolides apart from the aforementioned families of antibiotics: on average, the number of HBA and HBD are 15 and 4 respectively, the number of rotatable bonds is 9, the number of asymmetric carbons is 18 and the complexity index is 1260. Hence, macrocyclic lactone molecular flexibility is once again determinant for their

ribosomal target interactions compared to their efflux substrate qualities because 15-membered macrolides (Azithromycin) have more conformational degrees of freedom than 14-membered ones (Clarithromycin). Depending on the folded-out or the folded-in conformations, the polar groups of the lactone scaffold are directed towards one side of the molecule, while the other side is mostly hydrophobic [91]. Regarding the clear increase in MICs of 16-membered macrolides compared to 14- and 15-membered ones, it can therefore be assumed that the predominant bound conformation is the folded-out one which allows polar regions to bind to ribosomes, while the folded-in conformation could put the hydrophobic macrolide region (average LogP 9.09) in contact with the hydrophobic AcrB binding pocket. The number and position of sugar moieties have also an impact on the nature of the functional groups in the exposed conformation.

Aminoglycosides: Aminoglycosides are polycationic hydrophilic bactericidal antibiotics that affect protein synthesis by binding to the A-site 30S ribosome subunit. Their spectrum of action is broad, including *Enterobacteriaceae*, MRSA and mycobacteria. Resistance to Gram-positive bacilli has been observed, notably due to enzymatic transformation phenomena and 30S ribosome subunit mutation [92]. Gentamicin, Tobramycin and Amikacin, are the most used aminosides with minimal resistance in clinical practice because of accurate activity against Gram-negative aerobes strains [93]. Efflux phenomena are also occurring in resistance. The majority aminoglycoside efflux exporters belong to the RND pump family as AcrAB-TolC in *E. coli* or MexXY-OprM and MexAB-OprM pumps in *P. aeruginosa*. All these mechanisms may compromise the use of the whole class of agents [94]. The basic chemical structure required for the potency and spectrum of aminoglycosides activity is one or more amino sugars linked by glycosidic bonds to a 2-deoxystreptamine moiety (or streptidine in streptomycin derivatives). These molecules cross the outer membrane of the bacteria by disrupting magnesium Mg^{2+} bridges between lipopolysaccharide components. Their polycationic character due to the presence of 4 to 7 amino groups, and their pK_a around 9.6, favor this mechanism permeabilizing the external envelop and thus allowing the passage of materials [95]. Despite the highly polar property of amino groups, their large size does not allow crossing through porins. Passage across the inner membrane depends on a low Energy-Dependent Phase I electron transport, followed by a large Phase II influx requiring aminoglycoside-induced translation inhibition [96]. The particularly low LogP, up to -8.78 for Amikacin, favor antibiotic location in the periplasm. So, their uptake already shows the odd physicochemical profile from the other class of antibiotics that can cross the cytoplasmic membrane barrier spontaneously. Apart from the bacterial expression of enzymes which modify aminoglycoside structure, the main resistance mechanism which may affect antibiotics is precisely their decreasing uptake and/or accumulation. Aminoglycoside active efflux has long remained uncertain because not all RND pumps transport only lipophilic and amphiphilic substrates as previously seen. Indeed, AcrD is responsible for resistance to very hydrophilic aminoglycoside drugs. Compared to other antibiotics, aminoglycosides have a particularly high number of HBA and HBD, respectively 14 and 9 on average. In addition, the substrate specificity between AcrB and AcrD is different despite their strong sequence similarity [97] because it depends on the transporter domain location and thus the antibiotic uptake taking place mainly in the periplasm. Interactions with AcrD must be studied to identify molecular determinants to decrease efflux uptake without inducing AcrB efflux.

Other efflux substrates

Dyes: In general, dyes' structure is characterized by a tri or tetracyclic skeleton with 3 aromatic or heteroaromatic rings substituted by 1 or 2 aniline groups themselves functionalized by alkyl side chains. The succession of π-conjugated systems generates a planar skeleton with roughly flexible amino side chains by analogy with the structure of quinolones and cyclins. But their physicochemical properties such as the quantity of HBA and HBD, the amount of rotatable bonds, the number of stereocenters and the complexity are mostly lower than antibiotics which do not endow them with strong antibacterial potency. But the higher number of aromatic rings (3 to 5) and their amphiphilic character (LogP= -0.4, pK_a= 4,6) induce dyes substrates of RND pumps. They also have strong fluorescent properties when in contact with DNA (e.g., ethidium bromide) or membrane lipids (e.g., Nile red). So, variations in fluorescence can be checked in real time [98]. This approach produces quantitative data, allowing analysis of efflux kinetics in a way

that is impossible if we use bacterial growth-based efflux measurement methods such as MIC. This provides the opportunity to directly measure the consequences of environmental stressors such as antibiotic exposure on changes in bacterial efflux potential. These studies could highlight the mechanisms of interaction between bacterial cell responses and how they relate to changes in efflux pump function. As their overexpression is one of the major early triggers for the development of MDR phenotypes, this trend emphasizes the need to develop technologies and deploy diagnosis devices that provide meaningful (including efflux), rapid, and minimally invasive antibiotic susceptibility tests based on results relevant for therapeutic decision making while minimizing resistance phenomenon and thus therapeutic failure.

CONCLUSION

The crisis of anti-infective treatments is a public health problem that has become worse since the development of resistance is faster than that of new antimicrobials. As antibiotic treatments are still effective, the main challenge is to prevent the emergence of MDR pathogens. Physicochemical properties are at the foreground of study and practice in medicinal chemistry and are key descriptors that clearly affect drug attrition. In a drug discovery approach, physicochemical properties allow to define the noticeable molecular features that are related to interactions with different media and environments. Antibiotics are organic substances produced by living organisms, capable of inhibiting the growth of other microorganisms or of destroying them. Furthermore bacteria have developed resistance to antibiotics long before we started using them. This is exemplified by the β-lactam family with the successive discoveries of Penicillin G, Cephalosporin C, Thienamycin and Clavulanic Acid which have broadened the spectrum of antimicrobial activity to *P. aeruginosa* and enhance resistance to β-lactamases. Therefore, it is important to establish the environment-health link in epidemiological analyses of the AMR prevalence. Then, SAR studies allow characterizing a physicochemical profile important to improve pharmacokinetic-pharmacodynamic properties or to counteract resistance phenomena such as efflux. Although the causes of drug candidate attrition are multiple, analysis of drug design practices over the past decade shows that current molecules are favored with a high molecular weight and an unnecessary hydrophobicity. Indeed, the term "molecular obesity" [99] has been used to describe common hydrophobicity-oriented practices, and the excessive use of aromatic rings in structures. This is what we see with the quinolone or tetracycline family design containing fused aromatic rings or 4 adjacent cyclic hydrocarbon rings with high hydrophobicity conferring a rapid efflux resistance pace. Conversely, many findings in the drug research community have found their origin in macrocyclic natural products such as macrolides that occupy a unique physicochemical property space. Macrocyclic molecules can adopt biologically relevant conformations to bind their target, and the interactions can be optimized to achieve high potency and selectivity. Moreover, conformational variations can favor intramolecular and intermolecular bond network that make these compounds adaptable to their environment due to high hydrogen bond acceptor and donor counts that confer the profile so called "beyond the rule of 5" (bRo5) drugs [100]. Historically, bRo5 drugs have played an important role in the treatment of various pathologies, including infectious diseases and this trend has continued if we consider the evolution of the chemical structure of antibiotics, regardless of the family to which they belong [101]. As we have seen, the increase in flexibility, a higher amount of hydrogen bond acceptors and donors coupled with molecular complexity render antibiotics less subject to efflux and favor an intrabacterial concentration above the MIC and thus a greater efficacy. But bacteria also express RND pumps responsible for resistance, especially to hydrophilic drugs like aminoglycosides. If SAR studies have enabled us to understand how antibiotic can escape MDR efflux, they also permit to determine the molecular profile of their substrates. Indeed, well-chosen efflux substrates, such as dyes, would allow following the accumulation rate of the molecule according to the expression level of efflux pumps. This paves the way for the design of new diagnostic tools for the prevalence of bacterial efflux resistance in clinical practice.

CONFLICT OF INTEREST

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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DECLARATIONS

All authors whose names appear on the submission

1) made substantial contributions to the analysis and interpretation of data;

2) drafted the work and revised it critically for intellectual content;

3) approved the version to be published; and

agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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