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EDITED BY
Vinayak Singh,
University of Cape Town, South Africa

REVIEWED BY
Kushneet Kaur Sodhi,
University of Delhi, India
Abbas Yadegar,
Shahid Beheshti University of Medical Sciences, Iran

*CORRESPONDENCE
Sandrine Alibert,
sandrine.alibert@univ-amu.fr

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Molecular determinant deciphering of MIC-guided RND efflux substrates in *E. coli*

Johan Revol-Tissot [1,2], Gérard Boyer[3] and Sandrine Alibert [3]*

[1]Biotechnologie et Signalisation Cellulaire (BSC), UMR7242, CNRS, Strasbourg, France, [2]Institut de Recherche de l'Ecole de Biotechnologie de Strasbourg (IREBS), Université de Strasbourg, Strasbourg, France, [3]Aix Marseille University, INSERM, SSA, MCT, , Marseille, France

Antimicrobial resistance poses an urgent and formidable global public health threat. The escalation of bacterial multidrug resistance to antibiotics has the potential to become a leading cause of global mortality if there is no substantial improvement in antimicrobial development and therapy protocols. In light of this, it is imperative to identify the molecular determinants responsible for the reduced antibiotic activity associated with RND efflux pumps. This comprehensive study meticulously examines Minimum Inhibitory Concentration (MIC) data obtained from *in vitro* tests for various antibiotic families and non-active dye compounds, sourced from diverse literature references. The primary focus of this study is to assess the susceptibility of these agents to efflux-resistant *Escherichia coli* strains, integrating both MIC data and relevant physicochemical properties. The central objective is to unveil the specific substituents that significantly influence the uptake process mediated by the AcrAB-TolC efflux system. This exploration seeks to reveal the consequences of these substituents on pharmacodynamic responses, providing valuable insights into Structure-Activity Relationships. It is noteworthy that this analysis represents a pioneering effort, with prospective implications for RND efflux pump-producing strains. Ultimately, deciphering efflux markers is crucial to effectively mitigate the emergence of specific resistance and to better monitor the role of this primary resistance mechanism in Gram-negative bacteria, particularly as observed in clinical antibiotic therapy practice.

KEYWORDS

bacterial resistance, Gram-negative, *Escherichia coli*, RND efflux pumps, AcrAB-TolC, multidrug resistance, antibiotics, molecular determinants

1 Introduction

When antibacterials such as antibiotics and biocides began to be used to combat bacterial infectious diseases, it marked the onset of a new era in modern medicine. Their usage became widespread since 1945. Unfortunately, this new therapeutic arsenal has given rise to the reality of drug resistance.

The 2014 WHO report and the Review on Antimicrobial Resistance (AMR) highlighted disturbing epidemiological evidence regarding the acquired drug resistance of persistent bacterial pathogens in both hospitals and communities. These findings underscore the alarming projection that the death toll due to AMR could reach 10 million people annually by 2050. Recently, a study on the global burden of bacterial AMR was published, focusing on AMR-associated deaths in the year 2019 (Antimicrobial Resistance Collaborators, 2022).

Numerous health-environment investigations highlight the growing concern regarding antibiotic resistance, which stands as one of the leading causes of death worldwide (Sodhi and Singh, 2022; Singh et al., 2023; Sodhi et al., 2023). This concern is particularly pronounced as the estimated global death rate for 2050 has been reached over the past 5 years, surpassing 50 per cent of the predictions. These phenomena contribute significantly to the loss of antimicrobial activity for compounds that are still expected to be effective. Bacteria, like any living organism, continually adapt to their environment to survive and evolve over time and space (Muteeb et al., 2023). The molecular underpinnings of antimicrobial resistance emergence in bacteria trace their origins to antiquity. Pre-existing mechanisms can be considered as precursors to contemporary resistance determinants. These molecular pathways, strengthened through evolutionary processes, have endowed bacteria with the capacity to deploy survival strategies when confronted with natural antimicrobial agents from their environment (Christaki et al., 2020). This adaptive process has resulted in selective pressure and the emergence of antibiotic-resistant mutants in clinical contexts (Lerminiaux and Cameron, 2019; Smith et al., 2023).

The evolution of antibiotic resistance in common bacterial strains has extended to a wide range of antibiotics, contributing to the Multi-Drug Resistance (MDR) phenotype, especially in Gram-negative bacteria (Darby et al., 2023; Salam et al., 2023). This is evident in strains exhibiting acquired and intrinsic resistance mechanisms. They can be categorized in two main types, based on their nature and impact on antibiotic activity. The first type involves enzymatic degradation, particularly affecting β-lactams, aminoglycosides and some macrolides through pharmacophore hydrolysis or biotransformation, resulting in an inactive molecular structure (Tooke et al., 2019; Sawa et al., 2020). The second type encompasses intracellular target alterations in bacterial DNA and mRNA, affecting penicillin and quinolone derivatives in both Gram-positive and Gram-negative bacteria, leading to a poorly targeted molecule (Schaenzer and Wright, 2020; Spencer and Panda, 2023). These resistance mechanisms are specifically associated with one or two groups of antibiotics based on pharmacodynamic characteristics. They may also be coupled with other membrane effects that enable bacteria to extend the lag period in response during antibiotic exposure. At the membrane level, decreased porin expression (Vergalli et al., 2020) and, significantly, the overproduction of efflux pump are major contributors to the loss of effective intracellular antibiotic concentrations. These effects manifest early in exposure and involve all antibiotic classes, leading to the MDR phenotype. Consequently, they constitute the primary resistance barrier that initiates specific alternative mechanisms following exposure to toxic molecules (Krishnamoorthy et al., 2017).

Efflux pumps are ubiquitous protein complexes that actively extrude substances damaging to bacteria such as antibiotics, detergents, antiseptics, and toxins from the cytoplasm or periplasm into the external environment. These transporters recognize a wide range of physicochemically varied substrates (Blair et al., 2015; Zgurskaya et al., 2021). Currently, there are six 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 (Du et al., 2018; Hassan et al., 2018).

The last two classes, but more specifically RND transporters, which exhibit an energy-dependent tripartite system provided by a proton pump initially discovered in Tetracycline resistant *Escherichia coli*, were isolated similarly to P-glycoproteins in mammalian cells (Waghray and Zhang, 2018).

The intricate architecture of RND pumps comprises an elaborate assembly, including an inner membrane transporter, periplasmic adaptor proteins, and an outer membrane channel protein (Alav et al., 2021). These tripartite systems, prominently observed in Gram-negative bacteria, notably within the ESKAPE group, wield a pivotal role in conferring resistance. Their influence is particularly significant in the context of the escalating prevalence of antimicrobial resistance-related infections, standing as the primary cause of nosocomial infections on a global scale (Gauba and Rahman, 2023). The chief clinical concern associated with Resistance-Nodulation-Division (RND) pumps stems from their intrinsic linkage between expression levels and broad polyselectivity, evident in both the proximal and distal binding pockets of the inner membrane protein (Jang, 2023). This interconnection poses a formidable challenge in precisely identifying pharmacophore groups within the chemical structures of drugs implicated in the recognition and transport processes governed by these pumps.

Among the extensively studied efflux systems, AcrAB-TolC stands out as a focal point, particularly within enterobacteria such as *E. coli*, *Enterobacter spp.*, *K. pneumoniae*, and *K. aerogenes*. This well-explored system exhibits a diverse substrate profile encompassing various antibiotic classes, including chloramphenicol, tetracyclines, quinolones, macrolides, and β-lactams (Du et al., 2018). The elucidation of the intricate workings of these efflux systems is crucial for comprehending antibiotic resistance mechanisms and devising targeted strategies to counteract the rising tide of antimicrobial resistance in clinical settings. The AcrAB-TolC pump, a critical component in Gram-negative bacteria resistance, comprises three integral parts: the homotrimeric outer membrane channel TolC, the homotrimeric inner membrane transporter AcrB, and the periplasmic protein AcrA, present as six protomers, bridging the connection between these two integral membrane proteins (McNeil et al., 2019; Gaurav et al., 2023). State-of-the-art cryo-electron microscopy technologies, as employed by various research teams, have unveiled the intricate details of asymmetric trimeric structures characterizing AcrB. These studies span from seminal investigations (Murakami et al., 2006) to the latest advancements (El Omari et al., 2023), providing insights into a dynamic cyclic mechanism facilitated by a proton gradient. In this mechanism, each protomer assumes distinct conformations, aligning with the functional states of the transport cycle—Loose (L), Tight (T), and Open (O).

The peristaltic rotation mechanism, comprising three sequential steps, orchestrates the orderly binding of substrates within the periplasmic domain of one of the three protomers. This dynamic process has been elucidated through meticulous studies, highlighting a nuanced interplay of conformational changes (Tam et al., 2021). The structural intricacies and the complexities of substrate recognition and uptake render the AcrAB-TolC pump a

complex subject of study, further compounded by the involvement of intricate regulatory cascades in the expression of all RND efflux pumps. Notably in the context of *E. coli*, marA, ramA, and soxS emerge as robust activators responsible for inducing the expression of the AcrAB-TolC tripartite efflux pump systems. In the regulatory landscape, AcrR assumes the role of a specific repressor, playing a central and influential role in fine-tuning the expression of the associated efflux pump. Additionally, it is noteworthy that various bactericidal or bacteriostatic agents have been identified as potential triggers for this intricate regulatory cascade (Ferrand et al., 2020; De Gaetano et al., 2023). This multifaceted regulatory landscape adds another layer of complexity to the study of AcrAB-TolC, underscoring the need for comprehensive investigations to decipher the molecular determinants for efflux in the same way as regulatory elements.

Molecular modeling enables an assessment of the activity of new compounds using models that define physicochemical features connected with pharmacological and pharmacokinetic properties of bioactive agents. Notwithstanding the paramount significance of understanding the mechanisms governing substrate efflux for the enhanced design of antibiotics, an in-depth understanding remains elusive, primarily due to the extraordinary complexity inherent in these proteins (Athar et al., 2023). However, to limit efflux resistance outbreaks, it is imperative to understand the molecular determinants of the physicochemical properties involved in substrate uptake, particularly noteworthy with RND efflux pumps, which emerge as pivotal players in the context of antimicrobial resistance mechanisms (Li et al., 2015; Gil-Gil et al., 2023). Since the 90s, many efforts have been focused on studies on Structure Activity Relationships (SAR) of antibiotics. In most research papers, antibiotic activity is determined with Minimum Inhibitory Concentration (MIC), a valuable tool for diagnostics allowing clinicians to select the most appropriate antibiotic against a specific bacteria based on current and under development antibiotic susceptibility tests (Puttaswamy et al., 2018; Gajic et al., 2022). Nevertheless, only a small number of reviews consider the target site. As a result, their interpretation is less significant concerning efflux due to the diversity of membrane transporters, their complex component structure and function, particularly for RND pumps (Mouton et al., 2018). 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 based on their relevance to acquire a comprehensive array of antibiotics studied in connection with the same target. They are directly involved in the evolution of antibiotic structure compared to efflux resistance, aiming to promote an improvement in the use or design of antimicrobial agents. Additionally, the goal is to encourage further research on antibiotics and raise awareness within the scientific community about 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 enable the discovery of novel and improved antimicrobial chemical structures and the prevention of early resistance due to non-specific efflux impacts (Davin-Regli et al., 2021).

Antibiotics are integral to the therapeutic arsenal that has revolutionized the clinical treatment of infected patients. While some, like penicillin produced by the fungus *Penicillium*, tetracyclines, macrolides and aminoglycosides isolated from actinomycetes (Mullis et al., 2019), can be found in nature, others derived from natural products are semi-synthetic (e.g., ampicillin and amikacin) or fully synthetic (e.g., norfloxacin). They have specific actions on bacteria or protozoa, acting as bactericidal or bacteriostatic agents depending on their mechanism of action (Slonczewski and Foster, 2017; Halawa et al., 2024). The AcrAB-TolC archetype efflux pump represents a substantial database source in the scientific literature. We selected publications evaluating a broad range of antibiotics against Gram-negative efflux-resistant bacterial strains and matched the effects of AcrAB-TolC with 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. This pioneering study gives essential insight into various Gram-negative bacteria that produce AcrAB-TolC or other RND efflux pumps, significantly influencing drug activity at the target level without changing its multi-specific nature.

2 Review approach

We analyzed the pharmacological descriptor MIC, as it constitutes an appropriate index of bacterial strain resistance status and antibiotic sensitivity, along with several physicochemical properties. Among pharmacochemical descriptors, we used as structural parameters (Table 1) such as rotatable bonds, ring counts, including aromatic ones, stereocenter counts and complexity. As thermodynamic parameters, we considered octanol/water partition coefficient logarithm (LogP) and as electronic parameters, we included the 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 using software mentioned in the graph legend. Experimental physicochemical data were obtained for LogP and pK_a. Initially, LogP values were obtained for certain (fluoro)-quinolones and fluoro-naphthyridines (Ross and Riley, 1992; Takács-Novák et al., 1992; Hansch et al., 1995; Zhidong et al., 2006; Avdeef and Tam, 2010), β-lactams and cyclins (Sangster, 1993; Hansch et al., 1995; Al Bakain et al., 2011), macrolides (McFarland et al., 1997; Grabowski et al., 2010), aminoglycosides, and dyes (Hansch et al., 1995; Sarmah et al., 2006; Sanderson and Thomsen, 2009; Grabowski et al., 2010). Subsequently, using a similar method, pK_a values were obtained for certain (fluoro)-quinolones and fluoro-naphthyridines (Budavari et al., 1996; Torniainien et al., 1996; Park et al., 2000; Tolls, 2001; Hopfinger et al., 2009; Singh et al., 2009; Avdeef and Tam, 2010; Fujita et al., 2011), β-lactams (Sangster, 1993; Budavari et al., 1996; Khan and Ongerth, 2004; Huang and Hsieh, 2008; Fujita et al., 2011), cyclins (Serjeant and Dempsey, 1979; Avdeef and Tam, 2010; Fujita et al., 2011), macrolides (Budavari et al., 1996; McFarland et al., 1997; Qiang and Adams, 2004) and aminoglycosides (Budavari et al., 1996; Sarmah et al., 2006; Mucha et al., 2008; Fujita et al., 2011).

To enhance the comprehensibility of the MIC distribution based on antibiotics within the same family, we utilized boxplots

TABLE 1 Summary table of predicted or experimentally determined physicochemical properties of antibiotics and dyes included in this review.

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, pazufloxacin, 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)	[530/1,080] (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)	[740/1,250] (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, doripenem	[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, doxycycline chlortetracycline, eravacycline	[9/11] (9.8)	[5/7] (6)	[2/7] (3.8)	[971/1,240] (1,078.4)	[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)	[1,090/1,560] (1,260)	[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, streptomycin	[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 acceptors; ROT, rotatable bond counts; CMPX, complexity; STRC, stereocenter counts; R, ring counts; AR, aromatic ring counts.

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 (© 1994–2023 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).

(Figure 1). Each colored rectangle within these figures depicts the median MIC values, providing a clear representation of compound efficacies against the selected *E. coli* strains under investigation. Each examined antibiotic family is delineated within its respective quantitative statistical series for ease of interpretation. Moreover, this integrative approach not only facilitates cross-referencing pharmacological data with the physicochemical properties of each antibiotic, contingent on the added substituents as delineated in Table 1, but also ensures comprehensive elucidation. This meticulous presentation allows for a nuanced exploration of the relationships between pharmacological effectiveness and the structural attributes of the antibiotics under consideration.

Since *E. coli* is the most representative AMR pathogen and is frequently exposed to widely used antibiotics (Antimicrobial Resistance Collaborators, 2022), we studied the following efflux-resistant strains from literature MIC data, comparing them within a single antibiotic family (Figure 1A): 1) *marR* mutants: AG102 (point mutations correlated with the loss of *marR* repressor function by inhibiting *marO* promoter binding, leading to *marA* overexpression) (Cohen et al., 1988; Alekshun and Levy, 1997; Randall and Woodward, 2002; Whalen et al., 2015; Yilmaz et al., 2015; Vergalli et al., 2020; Ferrand et al., 2023), AG102B (Okusu et al., 1996; Aires and Nikaido, 2005) and 3-AG100 (Bohnert et al., 2008; Wehmeier et al., 2009; Bohnert et al., 2016; Schuster et al., 2016); 2) *marA* mutants: AG112 and CH164 (*marO* deletion mutation leading to an affinity loss with *marR* repressor and therefore *marA* overexpression) (Oethinger et al., 2000; Chollet et al., 2004; Nicoloff et al., 2006). Additionally, we selected some recombined strains with plasmids: HN1157/pHSGoxa7 with *acrR* deletion and penicillinase OXA-7 gene plasmid (operon *acrR* and *acrAB* transcription repressor) (Lim and Nikaido, 2010), GC7368/pCLL3431, an AG100A *E. coli* strain with cloned AcrAB gene leading to efflux pump overexpression (Visalli et al., 2003), JM101/pMAQ43, an MDR-derived strain with *ramA* (*acrA* overexpression in absence of *marA* or at least in reduced production) (George et al., 1995) and susceptible AG100A/pET28a-AcrB strain with *acrB* (plasmid containing the gene encoding AcrB leading to AcrAB-TolC overexpression as in

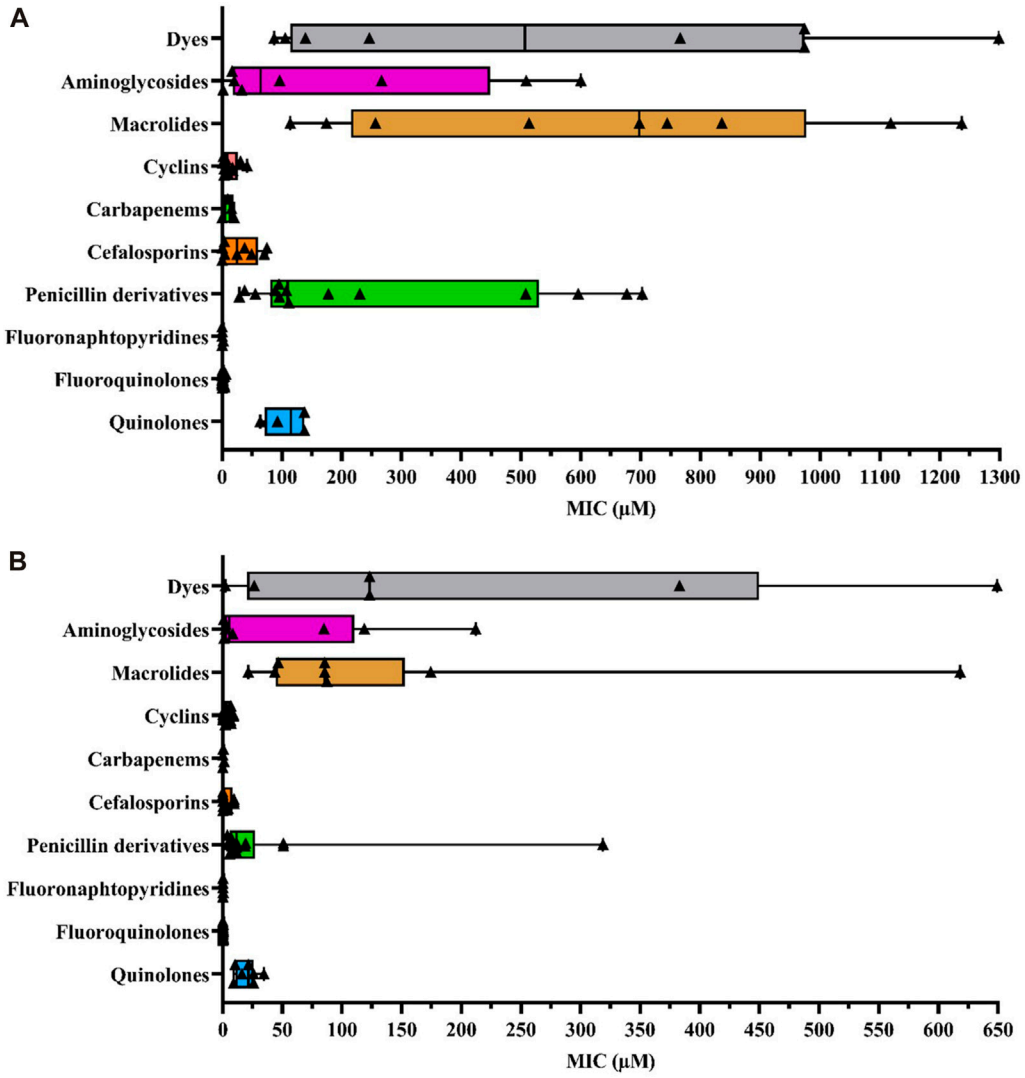

FIGURE 1
(A) Box plot representing MIC values according to different antibiotic families and other dye compounds in efflux-resistant *E. coli* strains **(B)** Box plot representing MIC values for different antibiotic families and other dye compounds in wild-type *E. coli* strains. The data points corresponding to each selected antibiotic, as extracted from the literature, are denoted by bold triangles (▲) for each family to construct the diagram. The statistical series include minimum, quartiles, mean, and maximal values of MICs (µM). Box plots were generated using GraphPad Prism 8.4.2 software.

GC7368/pCLL3431) (Li et al., 2011; Song et al., 2016). We also included clinical isolates with AcrAB-TolC overexpression, such as G5049 (Keeney et al., 2008; Sutcliffe et al., 2013). MIC data were collected from these efflux-resistant strains and compared between antibiotics and other agents to investigate variations in efflux response related to physicochemical properties.

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, such as: AG100 (Cohen et al., 1988; Okusu et al., 1996; Alekshun and Levy, 1997; Oethinger et al., 2000; Randall and Woodward, 2002; Visalli et al., 2003; Nicoloff et al., 2006; Bohnert et al., 2008; Keeney et al., 2008; Wehmeier et al., 2009; Yilmaz et al., 2015; Schuster et al., 2016; Song et al., 2016; Vergalli et al., 2020; Ferrand et al., 2023), ATCC25922 reference strain from EUCAST 2023 (Chollet et al., 2004; Aires and Nikaido, 2005; Lim Nikaido, 2010; Li et al., 2011; Sutcliffe et al., 2013; Whalen et al., 2015; Société Française de Microbiologie, 2023), JM101 (George et al., 1995), BW1556 (Munro and Kell, 2022) and BW25113 (Plé et al., 2022). Not all antibiotics have been assessed against strains overproducing AcrAB-TolC. Nevertheless, this strain provides the most exhaustive data regarding the wide range of tested antibiotic families compared to other AcrAB-TolC producer strains.

Thus, a SAR study can be defined for this purpose thanks to various structural parameter 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 concentrations ranging from 0.39 to 2 µM and 0.25–0.3 µM, respectively, compared to cyclins,

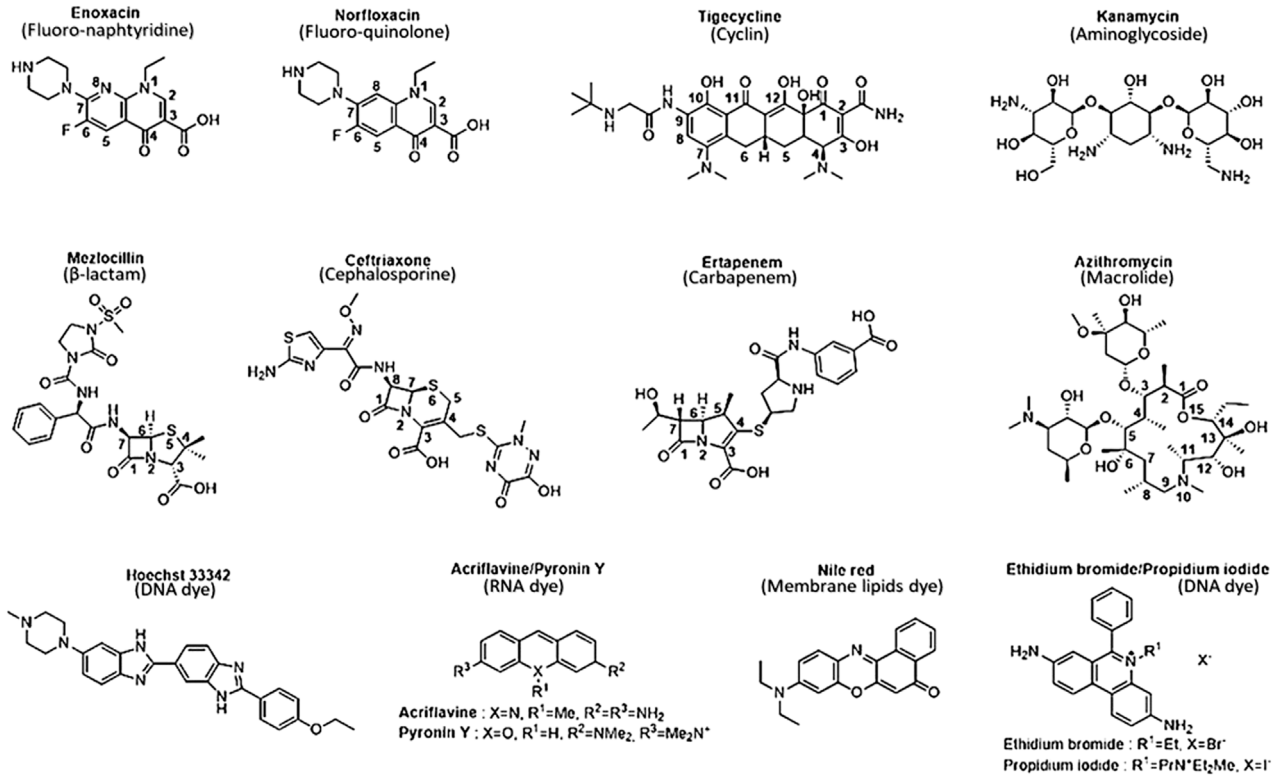

FIGURE 2
Chemical structure of the most representative antibiotics and dyes exhibit the best activity and weakest uptake by AcrAB-TolC efflux pumps. Selected antibiotics show lower MIC values in both wild-type and efflux-resistant *E. coli* strains, indicating heightened activity against resistant strains compared to wild-type strains. They possess physicochemical properties well-suited for strong activity against resistant-efflux strains, displaying optimal characteristics consistent with robust activity against such strains.

carbapenems, and cephalosporins. These 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), followed by carbapenems (15.6 μM), and finally cephalosporins (20.9 μM). Aminoglycosides display a narrower concentration range from 16.8 to 449.7 μM, which includes quinolones ranging from 85.4 to 137.8 μM. Penicillins have a wider concentration range from 88.9 to 537.5 μM, sharing a common feature with other antibiotic classes such as quinolones and aminoglycosides. Finally, macrolides represent the least active compounds in the overall *E. coli* series, with a MIC range between 247 and 982.9 μM. They have an activity almost twice as low as the least active penicillins. Thus, we find an overall disparity from 600 to 2,900 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).

Due to the repetitive testing of antibiotics on laboratory strains or clinical isolates, the scope of these compounds is constrained. Frequently, the same compounds are consistently chosen to assess efflux resistance in strains derived from patients or to compare the therapeutic efficacy of synthetic molecules with one or several existing antibiotics. To address this challenge, this review focuses on a carefully restricted selection of publications that present and provide a variety of antibiotics tested against a specific bacterial strain. This analytical approach aims to explore the connections between chemical structure and substrate properties in *E. coli*. Integrating data from other AcrAB-TolC-producing bacteria would yield limited insights, as each strain possesses the same efflux pump with the same substrate recognizing function. Essentially, if bacteria fail to efficiently assimilate a particular substrate, they respond by producing a novel efflux pump with a broader specificity. Consequently, variations may arise due to differences in membrane composition and expression levels, significantly influencing the duration of residence within the intracellular medium without altering the antibiotic's target affinity. The nature and composition of the targets also impose limitations in this comparison. Consequently, the molecular determinants involved in substrate recognition can vary due to alterations in amino acid composition and, more broadly, due to the distinct degrees of similarity among species that share a common efflux pump.

3 Discussions

3.1 Quinolone family

Quinolones represent the most powerful and widely used antibiotic family today. This class originated from nalidixic acid, the first quinolone discovered by accident as a by-product during the

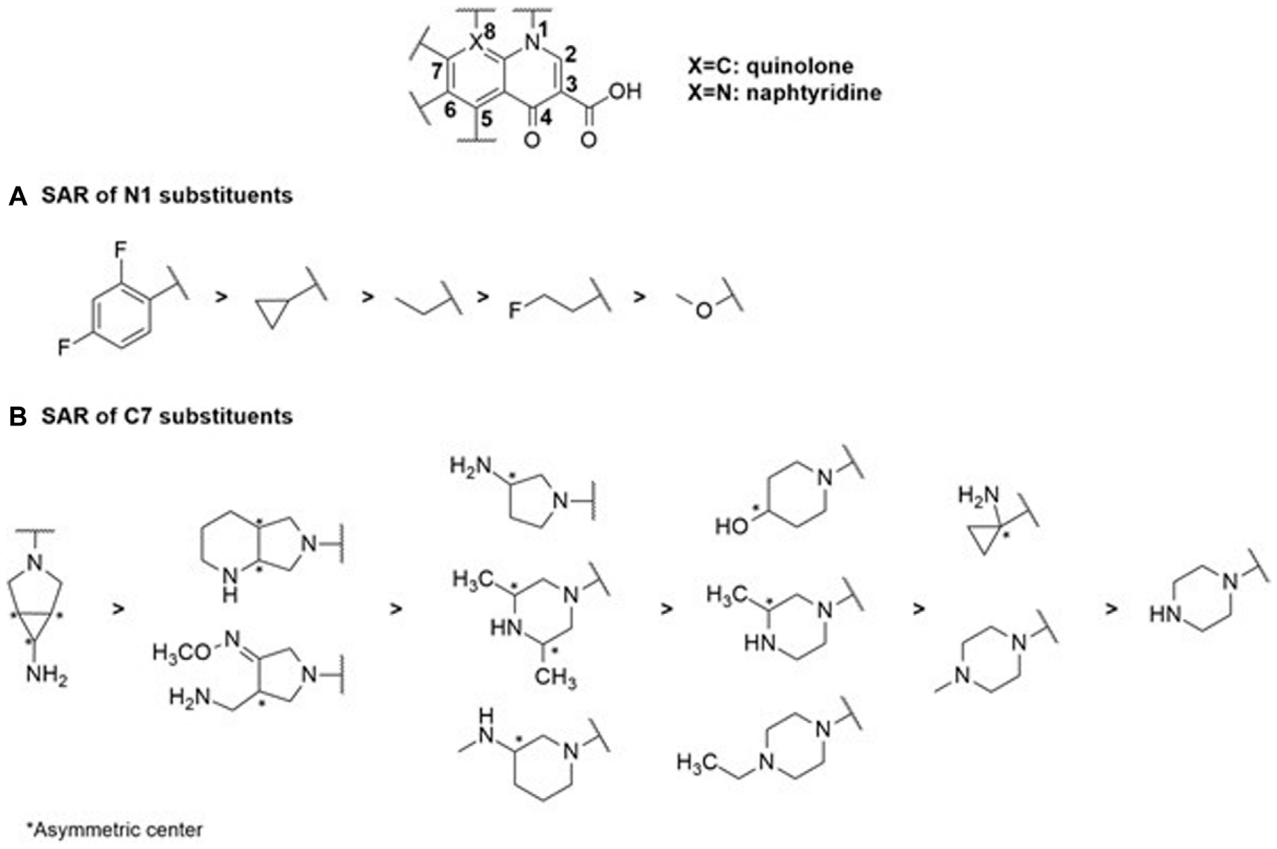

FIGURE 3
SAR study centered on the N1 **(A)** and C7 **(B)** substituents within the selected quinolone family of antibiotics reveals their substantial impact on MIC values and corresponding physicochemical properties.

synthesis of chloroquine (Lesher et al., 1962). Alongside other quinolones such as piromidic acid and cinoxacin, they constitute the first-generation quinolones. These bactericidal compounds act on DNA replication and mRNA transcription machinery in nuclear sites such as gyrases and topoisomerases, primarily for Gram-positive strains (Collin et al., 2011). The interaction of the enzyme with the molecule results in the formation of a stable complex between the antibiotic-bound enzyme and the DNA strands via covalent bonds (Klostermeier, 2021). Consequently, the double strand of DNA is broken, leading to the cessation of DNA replication and subsequent cell death. Although these first-generation quinolones lacked significant pharmacological activity against Gram-negative bacteria, they served as models for the synthesis of second-generation quinolones or fluoro-quinolones, which exhibit more substantial activities. Subsequent generations, such as ciprofloxacin and fleroxacin for second-generation quinolones or fluoro-quinolones, and levofloxacin and sparfloxacin for third-generation antibiotics, were synthesized based on their chemical structure. Moxifloxacin represents the latest generation (Dube et al., 2023). Despite their efficacy, all these compounds exhibit resistance phenomena often manifested through mutations at the level of gyrases and topoisomerases, resulting in a loss of affinity of fluoro-quinolones for their enzymatic target (Ezelarab et al., 2018). In Gram-negative bacteria, the efflux phenomenon is a major cause of resistance, particularly with the expression of RND-type pumps. These mechanisms, affecting many strains, lead to a restriction of their use over time and their enduring existence within the environment exerts a sustained influence as a driving force for natural selection (Krishnamoorthy et al., 2017; Bhatt and Chatterjee, 2023). However, recent developments in influx and efflux monitoring methods may aid in designing new fluoroquinolones, as demonstrated in the case of *E. coli,* and could potentially be extended to other *Enterobacteriaceae* and Gram-negative bacteria (Vergalli et al., 2020). In this context, a comprehensive study of a large set of 6-fluoro-quinolones and 6-fluoro-1,8-naphthyridines has been conducted in isogenic K12 strains of *E. coli* expressing different levels of AcrAB pumps. The results, particularly on the AG102 strain that overexpresses AcrAB, draw particular attention when comparing the impact of substituents in positions N1, C7, and C8 (Figure 2) on the MIC, which is inversely proportional to the level of efflux pump expression.

Firstly, it is noteworthy that there is no difference in activity regarding the structure of the 6-fluoro-quinolone series and the 6-fluoro-1,8-naphthyridines (Figure 2). For example, norfloxacin and enoxacin (Figure 2), which have the same functional groups, exhibit similar activity. The impact of changing the substituents at the C7 and C8 positions is also not significant. However, an additional

effect is observed related to the nature of the chemical function in position N1 (Figure 3). The MICs are markedly higher for compounds with aliphatic alkyl groups (ethyl in enoxacin, norfloxacin, lomefloxacin, pefloxacin, or fluoro-ethyl in fleroxacin) in this position or those forming a tricyclic structure with the C8 position (for pazufloxacin, ofloxacin, nadifloxacin) compared to cyclopropyl or difluoro-phenyl groups of the other molecules in the two series. These results indicate that the study of the SAR of the N1 chemical functions, crucial for the inhibition of the DNA gyrase, aligns with those obtained on a bacterial strain overexpressing efflux. Therefore, the N1 substituent appears to be a common pharmacophore for both gyrase and AcrB efflux pump targets. Alkyl functional groups are hydrophobic, and the decisive interaction site reported for AcrB substrates is a hydrophobic trap (Nishino et al., 2021), explaining the strong relationship between the N1 substituent and the efflux pump behavior of molecules. It is established that the AcrAB-TolC efflux pump is responsible for resistance to structurally unrelated antimicrobial agents (Lomovskaya et al., 2007; Zgurskaya et al., 2021) due to its broad substrate specificity (Alibert et al., 2017). However, increasing selectivity towards a therapeutic target requires ligand-target complementarity by limiting the molecular degrees of freedom to reach the "active" conformation. This can be achieved by rigidifying the molecular structure or inserting asymmetric centers (Figure 3). The overall number of rings and the number of aromatic rings tend to be less important for efflux-sensitive molecules. Additionally, the fewer asymmetric centers there are, the more the molecule's 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, therefore, 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 observed, no difference can be found in their scaffolds, so the molecular complexity lies in their substituents. Nalidixic acid (a naphthyridine with only alkyl groups in N1 and C7 positions) is particularly sensitive to efflux. Thus, increasing scaffold diversity could enhance the overall structural diversity of these types of antimicrobial agents. Diversity oriented synthesis could be the best solution to rejuvenate them.

3.2 β-lactam family

β-lactams constitute the oldest class of antibiotic employed in combating bacterial infections, encompassing various compound families such as penicillins, cephalosporins and carbapenems (Bush and Bradford, 2016). These molecules function by inhibiting peptidoglycan synthesis, a crucial process for membrane integrity that ensures the bacterial wall's structural integrity in response to external environmental pressures (Bugg and Walsh, 1992). In Gram-negative bacteria, peptidoglycan layers appear as a thin line within the periplasmic space and are cross-linked through the catalysis of transpeptidases, specifically penicillin-binding proteins (Park and Uehara, 2008). These enzymes become bound to a β-lactam, forming a highly stable penicilloyl-transpeptidase complex through the amide function of the β-lactam core (Figure 2). This core exhibits a structure closely resembling that of D-alanyl-D-alanine carboxypeptidase, a peptide located on the terminal chain of the peptidoglycan. As a result, the enzyme becomes obstructed and fails to fulfill its catalytic role. This obstruction leads to the destabilization of the bacterial wall, ultimately resulting in its degradation (Ghuysen et al., 1984).

3.2.1 Penicillins derivatives

Within this category of antibiotics, various classes have been developed. Penicillins were the primary β-lactam antibiotics used to target enzymatic degradations (Bradford, 2001). Initially effective against Methicillin Resistant *Staphylococcus aureus* (MRSA) strains and other Gram-positive bacteria, new compounds broadened the spectrum of action by enhancing the antibiotic's impact on certain Gram-negative bacteria through the synthesis of aminopenicillins (e.g., amoxicillin). Subsequently, the development of carboxypenicillins (e.g., ticarcillin) marked increased resistance to β-lactamases, expending the spectrum of action to include several enteric anaerobic bacteria and *P. aeruginosa* (Kaminski et al., 2011). In this context, the design of β-lactamase inhibitors has improved the stability of penicillins against enzymatic degradation (e.g., amoxicillin and clavulanic acid), enhancing activity against resistant pathogens (Bruyère et al., 2016). These inhibitors are valuable therapeutic agents, as many types of penicillins are no longer commonly used as monotherapy (Bush, 2018; Patel et al., 2019).

For the penicillin class of antibiotics, structural variations are only found in the C7 aminoacyl side chain of the penam scaffold (Figure 4). Its length is variable, featuring 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 exhibit the highest MIC. The higher number of aromatic rings in them gives a greater capacity to be substrates of efflux due to the AcrB binding site rich in aromatic amino acids. Moreover, all the molecular fragments constituting it are conjugated, conferring coplanarity and thus a weak flexibility characterized by a low number of rotatable bonds (on average 5). The SAR study indicates that oxazole pharmacomodulation strategy, which improved the absorption of these antibiotics and their resistance to β-lactamase, has increased efflux resistance compared to penicillins G and V, ampicillin, and amoxicillin. Finally, penams with the lowest level of efflux transport and uptake are those with polyamide chains (Figure 4). However, the greater flexibility of penams (up to 6 rotational bonds) is relative, due to the succession of π-π-*n* conjugate systems that block free rotations around the amide bonds, reinforced by their cyclisation. A higher amount of HBA and HBD, greater polarizability, and a higher number of asymmetric carbons enhance the penam molecular complexity, contributing, along with lower LogP and pK_a, to reduce sensitivity to efflux while providing better specificity towards their therapeutic target compared to the hydrophobic AcrB. As mentioned above, increased scaffold diversity allows increased drug potency.

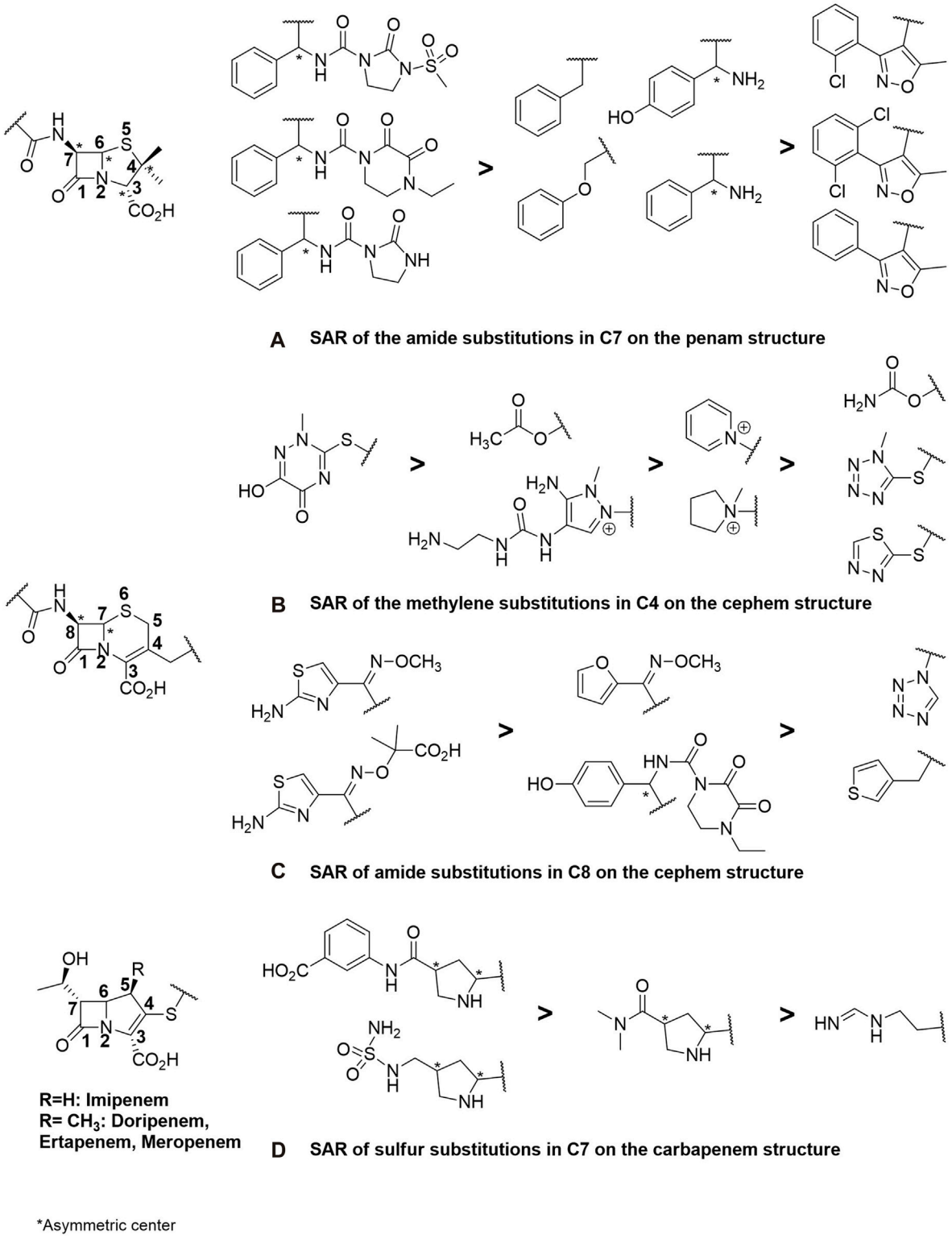

FIGURE 4
SAR study encompassing substitutions at strategic positions, specifically focusing on **(A)** the C7 amide in penam (penicillin derivatives), **(B,C)** C4 methylene and C8 amide in cephem (cephalosporins), and finally, **(D)** the C7 sulfur carbapenem structures included in theβ-lactam antibiotics.

3.2.2. Cephalosporins

Indeed, the discovery of cephalosporins has expanded the spectrum of activity of antibiotics. The incorporation of the β-lactam ring with a dihydrothiazine instead of a thiazolidine ring has allowed the introduction of a new substitution position in the structure, increasing molecular diversity. Structural enhancements

have given rise to five generations of antibiotics. The first generations (e.g., cefuroxime) exhibited resistance to MRSA and some *Enterobacteriaceae* (*E. coli*, *K. pneumoniae*) and subsequently to β-lactamases (Ghuysen et al., 1984). However, amendments were made from the third generation onward (e.g., cefotaxime, cefepime) because, despite decreased activity against staphylococci and enterococci, they demonstrated lower β-lactamase affinity and a broader spectrum of action against Gram-negative bacteria and MRSA (Bush, 2015). The latest generation of cephalosporins, known as ceftolozane, is currently used in combination with β-lactamase inhibitors for highly potent activity against enzymes overproducing pseudomonal pathogens (Zhanel et al., 2014). Cefalexin, in fact, was prepared from the sulfoxide ester of the corresponding penicillin by ring expansion (Garbrecht, 1972). In general, cephems exhibit better antibacterial activity than penams, a fact confirmed in efflux-resistant *E. coli* strains. Cefazolin and cefoxitin share structural analogies with penicillins such as mezlocillin (Figure 2), piperacillin, and azlocillin, including the length and flexibility of their side chains and the number of HBA and LogP. The insertion of an O-substituted oxime function (Figure 4) has allowed for the lengthening and cross-linking of the side chain of third and fourth-generation cephalosporins, significantly reducing the MIC by enhancing the flexibility (8 rotatable bonds) of these antibiotics and the number of HBA (10–13). This results in higher molecular complexity (740–1,250), which strongly contributes to decreasing hydrophobicity and, consequently, sensitivity to efflux, leading to better inhibition of cell wall biosynthesis and enhanced bactericidal effects.

3.2.3 Carbapenems

Carbapenems share similar structural characteristics, but they also exhibit differences that contribute to their broad-spectrum activity, high potency, and classification as last-resort antibiotics. Derived from penicillin through 3-position substitutions in thiol groups (Figure 4), the most representative among them is imipenem, employed in cases of resistance of Gram-negative bacilli against the third generation of cephalosporins due to enzyme overproduction (El-Gamal et al., 2017). Resistance to carbapenems can also be observed in MRSA and the emergence of Carbapenem-Resistant *Enterobacteriaceae,* generated by the synthesis of a specific group of β-lactamases called carbapenemases. This takes into consideration the escalating impact of stress-induced upregulation in AcrB expression and efflux pumps, notably triggered by imipenem in *E. coli* (Iovleva and Doi, 2017; Chetri et al., 2019). The latest widely available antibiotic is doripenem, known for its higher chemical enzyme stability and potency against Gram-negative bacteria (Nordmann et al., 2011). Firstly, their side chains differ from those found in penams and cephems, featuring 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, which begins with a sulfur atom (Figure 4). Hydrogen atoms at the C6 and C7 positions have a *trans* configuration compared to the *cis* configuration of penicillins and cephalosporins (Figure 2). Although the complexity index of carbapenems remains in the same order as that of other β-lactams, the higher average number of asymmetric carbons (up to 6 carbons, Figure 4), the absence of aromatic rings (except one for ertapenem, Figure 4), and very low hydrophobicity (on average −1.66 against −0.90 and 1.45, respectively, for cephems and penams) endow them with a significantly low efflux substrate character. This, in turn, enhances the duration during which the intrabacterial antibiotic concentration surpasses the MIC. These physicochemical and structural properties can be considered as the parameters that best correlate with the efficacy of antimicrobial agents.

3.3 Cyclin, macrolide, aminoglycoside families

Cyclins, macrolides, and aminoglycosides act by inhibiting protein synthesis through interactions with ribosome subunits. This step is crucial for protein biosynthesis following mRNA translation (Macé et al., 2015).

3.3.1 Cyclins

Cyclins belong to a class of antibiotics derived from tetracycline that can penetrate eukaryotic cells. This feature makes them suitable for targeting intracellular parasitic bacteria. These compounds are bacteriostatic and operate by inhibiting the 30S ribosome subunit through the inhibition of tRNA-A-site interaction. Their spectrum of action extends to combating both Gram-negative and Gram-positive bacteria, including naturally resistant *Enterobacteriaceae*, anaerobic pathogens, and *Pseudomonas aeruginosa*. Doxycycline remains the most widely used and clinically recommended therapeutic agent (Grossman, 2016). Unfortunately, mutations in the ribosomal complex, as well as efflux resistance phenomena due to their bacteriostatic activity, have contributed to the emergence of mutants with overexpression of RND (*mar* locus mutation) and MFS (*tet* protein) family pumps. Ribosomal mutations, efflux resistance, and ribosomal protection through a greater tetracyclines-A-site dissociation constant (K_D) have significantly reduced the pharmacological activity of this class of antibiotics, both for past and novel derivatives (Chopra and Roberts, 2001; Warburton et al., 2016).

Compounds within the tetracycline family share a common octahydro-naphthacene-carboxamide basic structure (Figure 5), crucial for their activity. Enolized tricarbonyl-methane and phenolic diketone systems are present at positions C1-C3 and C10-C12, respectively (Figure 2). Their amphoteric behavior is attributed to the dimethylammonium group at C4 (Figure 5). Variations in activity are particularly attributed to substitutions at positions C6, C7, and C9 (Figure 5). Favorable substitutions include the replacement of the aromatic ring with a dimethylamino or fluoro group at C7 and an aminoalkyl chain branched on an acylamino function at C9. Like other antibiotic families, the presence of a long flexible chain of nitrogenous functions significantly reduces the substrate character of AcrB-type efflux pumps in *E. coli* (by a factor of 5 between the MIC of minocycline and tigecycline (Figure 2), for example,). Decisive physicochemical parameters include the number of HBA and HBD (optimum at 11 and 7, respectively), the number of rotational bonds (optimum at 7), and the complexity index (>1,000). In the case of tetracyclines, the number of stereocenters, LogP, and the number of rings, particularly aromatic rings, are less significant due to the common nature of the octahydro-naphthacene scaffold known to

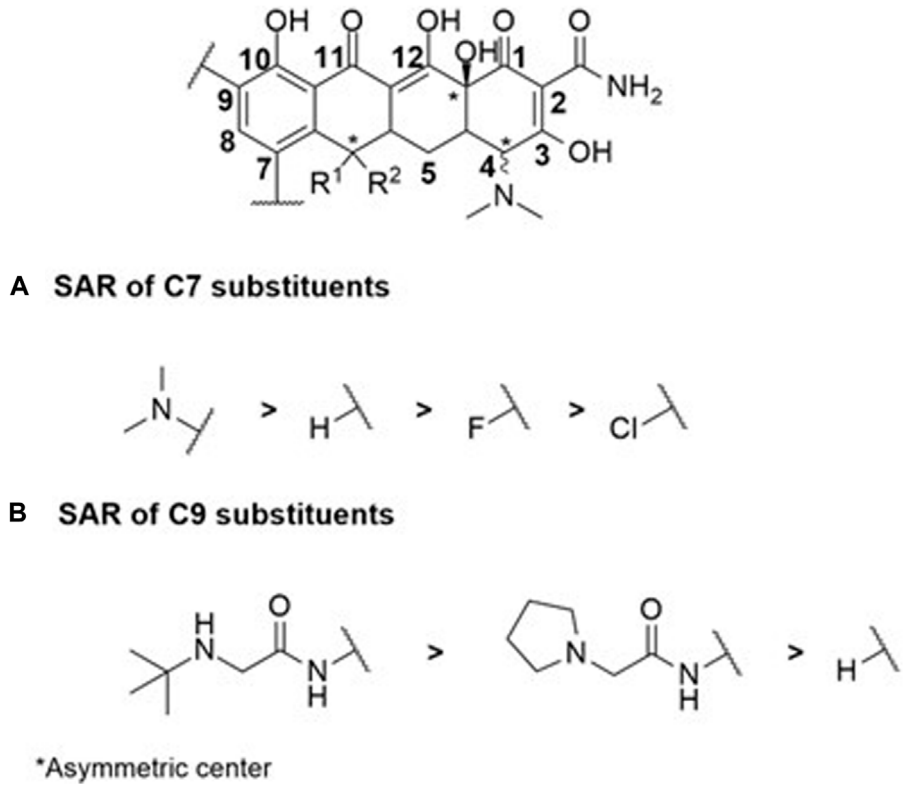

FIGURE 5
SAR study centered on C7 **(A)** and C9 **(B)** substituents within the octahydro-naphthacene-carboxamide moiety illustrates the tight correlation between the physicochemical properties of the substituents and antibiotic biological activity among the studied cyclin antibiotics.

interact with various pharmacological targets. Co-crystallization of doxorubicin and minocycline in a putative AcrB binding pocket suggests that the broad substrate spectrum of AcrB results from interactions with hydrophobic amino acids [e.g., phenylalanine residues F136, F178, F610, F615, F617, and F628 (Murakami et al., 2006; Nakashima et al., 2011)] and, to a lesser extent, polar residues in the binding site. Therefore, even with improvements to its basic pharmacophore, this antibiotic class would remain limited by AcrAB-TolC uptake, necessitating the exploration of other synthesis strategies for chemical optimization.

3.3.2 Macrolides

Macrolides are bacteriostatic compounds that operate by inhibiting the 50S ribosome subunit, dissociating it from tRNA. They exhibit a medium spectrum of action and demonstrate broad resistance against *Enterobacteriaceae*, *Pseudomonas,* and some Gram-positive bacilli due to methylation of the 23S ribosomal RNA subunit. The classes of compounds vary from 14 atoms (e.g., erythromycin) to 16 atoms (e.g., josamycin). Their activity is compromised by slow accumulation in Gram-negative bacteria, attributed to low permeability, not overlooking the intervention of RND and ABC efflux pumps, which significantly reduces the effective intracellular concentration of these antibiotics (Zhong and Shortridge, 2000; Kobayashi et al., 2001; Vázquez-Laslop and Mankin, 2018). Despite the distinctive physicochemical and molecular characteristics that set macrolides apart from other antibiotic families—with an average of 15 HBA and 4 HBD, 9 rotatable bonds, 18 asymmetric carbons, and a complexity index of 1,260—macrocyclic lactone (Figure 2) molecular flexibility remains crucial for their ribosomal target interactions compared to their efflux substrate qualities. Notably, 15-membered macrolides (such as azithromycin, Figure 2) have more conformational degrees of freedom than 14-membered ones (such as 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 (Jednačak et al., 2020). Considering the evident increase in MICs of 16-membered macrolides compared to 14- and 15-membered ones, it can be assumed that the predominant bound conformation is the folded-out one, allowing polar regions to bind to ribosomes. Conversely, the folded-in conformation could bring the hydrophobic macrolide region (average LogP 9.09) into contact with the hydrophobic AcrB binding pocket. The number and position of sugar moieties (Figure 6) also impact the nature of the functional groups in the exposed conformation.

3.3.3 Aminoglycosides

Aminoglycosides are polycationic hydrophilic bactericidal antibiotics that impact 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

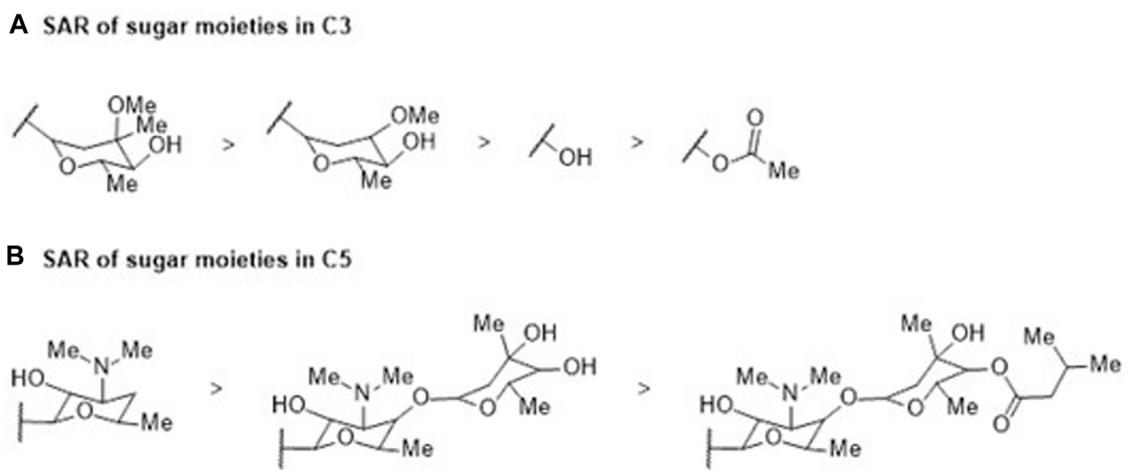

FIGURE 6
SAR study involving **(A)** C3 and **(B)** C5 sugar moieties within the macrocyclic lactone structure of the reviewed macrolide antibiotics. Variations in activity are indicated in relation to the incorporation of a sugar moiety, which subsequently influences the physicochemical properties.

enzymatic transformation phenomena and 30S ribosome subunit mutation (McDermott et al., 2003; Ojdana et al., 2017). Gentamicin, tobramycin and amikacin are the most widely used aminoglycosides with minimal resistance in clinical practice due to their accurate activity against Gram-negative aerobic strains (Lang et al., 2023). Efflux phenomena also occur in resistance. Most aminoglycoside efflux exporters belong to the RND pump family, such 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 entire class of agents (Poole, 2005; Thacharodi and Lamont, 2022). 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 4–7 amino groups, and their pK_a around 9.6, favor this permeabilization mechanism of the external envelope, thus allowing the passage of materials (Vaara, 1992). Despite the highly polar property of amino groups, their large size does not allow them to cross 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 (Davis, 1987). The particularly low LogP, up to −8.78 for amikacin, favors the antibiotic's location in the periplasm. Thus, their uptake already demonstrates an unusual physicochemical profile compared to other classes of antibiotics that can spontaneously cross the cytoplasmic membrane barrier. Apart from the bacterial expression of enzymes modifying aminoglycoside structure, the main resistance mechanism affecting antibiotics is precisely their decreased uptake and/or accumulation. Aminoglycoside active efflux has long remained uncertain because not all RND pumps transport only lipophilic and amphiphilic substrates, as seen previously. 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 (Elkins and Nikaido, 2002) because it depends on the transporter domain location, and thus, the antibiotic uptake mainly takes place in the periplasm. Interactions with AcrD must be studied to identify molecular determinants to decrease efflux uptake without inducing AcrB efflux.

3.4 Other efflux substrates: dyes

In general, the structure of dyes (Figure 2) 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 relatively flexible amino side chains, analogous to the structure of quinolones and cyclins. However, their physicochemical properties, such as the quantity of HBA and HBD, the number of rotatable bonds, the number of stereocenters, and the complexity, are generally lower than antibiotics, which does not endow them with strong antibacterial potency. Nevertheless, the higher number of aromatic rings (3–5) and their amphiphilic character (LogP = −0.4, pK_a = 4,6) make dyes substrates for RND pumps (Murakami et al., 2006; Nakashima et al., 2011). They also exhibit strong fluorescent properties when in contact with DNA (e.g., ethidium bromide) or membrane lipids (e.g., Nile red). Thus, variations in fluorescence can be monitored in real time (Iyer and Erwin, 2015; Blair et al., 2016). This approach yields quantitative data, enabling the analysis of efflux kinetics in a manner impossible with bacterial growth-based efflux measurement methods such as MIC. It provides the opportunity to directly measure the consequences of

environmental stressors, such as antibiotic exposure, on changes in bacterial efflux potential (Salcedo-Sora et al., 2021). These studies could elucidate the mechanisms of interaction between bacterial cell responses and their relationship to changes in efflux pump function. As the overexpression of efflux pumps 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 phenomena and, thus, therapeutic failure. In this methodology, it is imperative to devise and/or identify one or more inert molecules capable of being effluxed by any AcrAB-TolC-producing bacterium or other RND pumps. This approach maintains efflux pump uptake as a universal parameter, allowing manipulation solely of the interaction and specificity between an antibiotic molecule and its target. This ensures the preservation of the excretory function, adaptable to diverse structural patterns. Consequently, dyes can emerge as potent instruments for researchers and clinicians, facilitating comprehension and adaptation of antimicrobial treatments to address efflux-related challenges and enhance patient care.

4 The clinical impact of RND efflux on AMR

Most of the clinically studied strains exhibit specific resistance combined with efflux mechanisms (Davin-Regli et al., 2021). However, efflux is typically not investigated in epidemiological studies involving clinical strains. Studies conducted in the laboratory show that inhibiting efflux mechanisms never fully restores their susceptibility to antibiotics, despite a significant reduction in MIC.

AcrAB is the major multidrug-resistant (MDR) pump in *E. coli*. This pump exhibits constitutive expression in wild-type strains and undergoes substantial overexpression during exposure to drugs (Teelucksingh et al., 2020). Additionally, close homologs such as AcrD, AcrEF, MdtABC, and YhiUV (MdtEF) play a secondary role, triggered only after AcrB inactivation (Anes et al., 2015). Notably, MdfA transporters, linked to quinolone resistance, belong to the MFS family (Yasufuku et al., 2011).

Despite the clinical importance of *E. coli*, studies on the role of efflux in hospital infections are limited. A study in Japan in 2008 revealed a correlation between overexpression of the marA gene, upregulating acrAB, and resistance to fluoro-quinolones (Yasufuku et al., 2011). Another study in China in 2018 found high resistance rates in *E. coli* strains isolated from urine samples, with increased expression of various efflux pump genes, including ABC transport efflux pump genes and acrB (Zeng et al., 2020).

In an international collection of MDR *E. coli*, Camp et al. (2021) explored efflux mechanisms using MIC tests with an AcrAB-TolC efflux inhibitor and qRT-PCR. Their findings indicated that 50% of strains had efflux mechanisms associated with antimicrobial resistance. Significant overexpression of the AcrAB-TolC system was observed in these strains, supported by whole-genome sequencing showing amino acid substitutions in genetic regulators AcrR, MarR, and SoxR.

In essence, the evidence presented underscores the crucial role of efflux pumps, particularly AcrAB, in mediating resistance in *E. coli*, and emphasizes the need for further research in clinical settings to understand and address the implications of efflux mechanisms in antibiotic resistance.

5 Conclusion

The crisis in anti-infective treatments exacerbated by the rapid development of resistance compared to the creation of new antimicrobials, presents a significant public health concern. While current antibiotic treatments remain effective, the primary challenge is to prevent the emergence of MDR pathogens.

Historically, medicinal chemists have sustained the antibiotic arsenal by synthetically adapting existing antibacterial structures to counteract evolving resistance. The evolution of β-lactam antibiotics into multiple generations exemplifies this method. Modification of existing structures remains the primary strategy for managing antibiotic resistance today. However, a long-term strategy for managing antibiotic resistance is essential to regain our medicinal advantage. With the increase in antibiotic resistance and the impact of broad-spectrum antibiotics, the development of specific antibiotics has become more attractive, especially when combined with rapid diagnostics.

Physicochemical properties play a crucial role in medicinal chemistry, serving as key descriptors that significantly impact drug attrition. In drug discovery, physicochemical properties define molecular features related to interactions with different media and environments.

Antibiotics, organic substances produced by living organisms, inhibit the growth of other microorganisms. Bacteria have developed resistance to antibiotics long before their human use, exemplified by the successive discoveries in the β-lactam family. Penicillin G, cephalosporin C, thienamycin and clavulanic acid broadened the spectrum of antimicrobial activity to include *P. aeruginosa* and enhanced resistance to β-lactamases. The environment-health link in epidemiological analyses of the AMR prevalence is crucial. Natural products play a major role in drug discovery, with half of therapeutic small molecules having a chemical structure based on a natural product. This impact is even more significant for certain therapeutic classes, such as antibiotics.

Natural products exhibit molecular scaffolds evolved to bind to biological targets, resulting in complex structures with physicochemical characteristics correlated with increased binding specificity, reduced preclinical toxicity, and improved pharmacokinetic profiles. SAR studies characterize a physicochemical profile important for improving pharmacokinetic-pharmacodynamic properties or countering resistance phenomena such as efflux.

While the causes of drug candidate attrition are multiple, an analysis of drug design practices over the past decade demonstrates that recent molecules favor high molecular weight and unnecessary hydrophobicity. “Molecular obesity” (Hann, 2011) describes common hydrophobicity-oriented practices, such as the excessive use of aromatic rings in structures seen in the design of quinolones or tetracyclines, containing fused aromatic rings or four adjacent cyclic hydrocarbon rings, leading to rapid efflux resistance.

Conversely, some findings trace their origin to macrocyclic natural products such as macrolides with unique physicochemical properties, favoring intramolecular and intermolecular bond networks allowing conformational variations that optimize interactions for high potency and selectivity. This chemical adaptation to their environment is due to the high number of HBA and HBD, conferring the so-called "beyond the rule of 5" (bRo5) drugs (Danelius et al., 2020). Historically, bRo5 drugs have played an important role in the treatment of various pathologies, including infectious diseases and this trend has continued with the evolution of the chemical structure of antibiotics, regardless of the family to which they belong (Degoey et al., 2018). There is growing interest in exploring bRo5 drugs, including natural product-based drugs exhibiting lower hydrophobicity and higher stereochemical content compared to their purely synthetic counterparts. This pharmaceutically relevant chemical space offers increased structural diversity for drug discovery. Increased flexibility, higher HBA and HBD, coupled with molecular complexity make antibiotics less prone to efflux and enhancing intrabacterial concentration above the MIC and efficacy. However, bacteria express RND pumps responsible for resistance to hydrophilic drugs like aminoglycosides. SAR studies have elucidated how antibiotics can escape MDR efflux, providing insights into molecular profile of efflux substrates. Dyes, chosen as efflux substrates, could enable the monitoring of molecule accumulation based on efflux pump expression levels. This paves the way for designing new diagnostic tools for assessing bacterial efflux resistance prevalence in clinical practice, following a one-health approach.

This review aims to establish a relevant model correlating SAR studies on AcrAB-TolC- mediated efflux with inherent structure-property relationships, considering additional molecular determinants and their implications for antimicrobial and substrate activity. The study focuses on AcrAB-TolC and *E. coli,* emphasizing the significance of enhancing the antibiotic affinity for its target, as the pivotal factor then becomes the speed at which it engages with the target. Efflux, prevalent in all Gram-negative bacteria through various RND pumps, remains nonspecific. This approach offers a pathway to enhance the effectiveness of upcoming antibiotics by scrutinizing existing ones where efflux poses a substantial hindrance to pharmacological activity.

In conclusion, as we confront the formidable challenge of antibiotic resistance, it becomes increasingly apparent that a multifaceted approach is essential in shaping the future of antibiotic development. Beyond the considerations discussed, such as physicochemical properties, natural product-based drugs, and efflux mechanisms, several other critical aspects demand attention.

Targeted drug delivery holds promise in optimizing the efficacy of antibiotics while minimizing systemic exposure and side effects. Combination therapy, by exploring synergistic combinations of antibiotics or combining them with other therapeutic agents, offers avenues to enhance effectiveness and combat resistance. Host-directed therapy presents an opportunity to modulate host immune responses to augment infection clearance and antibiotic efficacy.

The integration of nanotechnology enables innovative strategies for drug delivery, pharmacokinetics optimization, and stability enhancement, further advancing the effectiveness of antibiotics. Surveillance and monitoring systems are indispensable for tracking antibiotic resistance patterns and identifying emerging threats in real-time, while regulatory frameworks play a pivotal role in incentivizing the development of new antibiotics and ensuring their timely approval and access.

By addressing these challenges and embracing innovative strategies, we can forge a path toward more effective and sustainable treatments to combat the growing threat of antibiotic-resistant infections.

Author contributions

JR-T: Writing–original draft. GB: Writing–original draft. SA: Writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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