



Medicinal Chemistry Updates on Bacterial Efflux Pump Modulators

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Abstract: Antibiotic resistance is one of the most pressing health issues of our days. It can arise due to a multiplicity of factors, such as target modification, decrease in the drug uptake, changes in the metabolic pathways and activation of efflux pumps. The overexpression of efflux pumps is responsible for the extrusion of drugs, making antibiotic therapy fail, as the quantity of intracellular antibiotic is not enough to provide the desired therapeutic effect.

Efflux pumps can be included in five families according to their composition, nature of substrates, energy source, and number of transmembrane spanning regions. The ABC superfamily are mainly found in Gram-positive bacteria, use ATP as an energy source, and only a limited number of ABC pumps confer multidrug resistance (MDR).

On the other hand, the MFS family, most present in Gram-positive bacteria, and the RND family, characteristic of Gram-negative bacteria, are the most associated with antibiotic resistance. A wide variety of inhibitors have been disclosed for both families, from either natural or synthetic sources, or even drugs that are currently in therapy for other diseases.

The other two families are the SMR, which are the smallest drug efflux proteins known, and the MATE family, whose pumps can also resort to the sodium gradient as an energy source.

In this review, it is intended to present a comprehensive review of the classes of efflux pump inhibitors from the various sources, highlighting their structure-activity relationships, which can be useful for medicinal chemists in the pursuit of novel efflux pump inhibitors.

Keywords: Antimicrobial resistance; efflux pump inhibitors; natural products; synthetic compounds; existing drugs; structure-activity relationship.

1. INTRODUCTION

Bacteria were discovered the late 19th century as infectious agents. It was, however, half a century later that antibiotics completely revolutionized medicine and became a turning point in human history. Unfortunately, the misruled use of these drugs led to the appearance of resistant strains, and to the concern of a return to the pre-antibiotic era, where the simplest, most treatable infections can become deadly [1-2].

Antibiotic resistance can arise due to multiple causes. The most common are the overuse and/or inappropriate prescribing, as well as extensive veterinary and agricultural use. These factors are especially alarming if it is taken into account the very few new antibiotics that were discovered in the past years [3]. Due to their genetic plasticity, bacteria can develop defense mechanisms in response to environmental threats. As far as antibiotics are concerned, bacteria use two major strategies to adapt to these drugs, which are mutations in genes and the acquisition of resistance genes through horizontal gene transfer. The first strategy can be manifested through modification of the target, decrease in the drug uptake, activation of efflux pumps to extrude the drug or changes in metabolic pathways. The latter is related to the acquisition of external genetic material through transformation, transduction, or conjugation [4-5]. Recently, vesicle-mediated resistance has also been regarded as a mechanism of resistance acquisition [6].

Efflux pumps are ubiquitous in bacteria, and can be encoded by genes present either in the bacterial chromosomes or in plasmids [7-8]. They concern five families, according to their composition, substrates, energy sources, and number of

transmembrane spanning regions: the adenosine triphosphate (ATP)-binding *cassette* (ABC) superfamily, the resistance-nodulation-division family (RND), the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family (**Figure 1**) [7-12].

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Efflux pumps can be further divided into two major families, the primary transporters, which hydrolyze ATP as a source of energy, as is the case of the ABC family, and secondary transporters, which use the proton or sodium gradient as a source of energy [13]. Overexpression of multidrug efflux pumps is correlated with an increase in the minimum inhibitory concentration (MIC) of antibiotics, and thus, these pumps can be considered potential antibacterial targets.

Therefore, the development of efflux pump inhibitors may be a way of fighting against resistant microorganisms. Studies have been carried out on the structure of efflux pumps, as well as on their mechanisms of binding and extrusion, leading to the possibility of structure-based design of efflux pump inhibitors (EPIs). The rationale for the development of these EPIs could be, for instance, the inhibition of the energy sources required for the ability to extrude antibiotics, and the development of compounds that compete with the antibiotics for their extrusion. However,

the main challenge lies in the fact that many compounds that present EPI activity and are already used for the treatment of other diseases are toxic for human cells in the concentrations required to present EPI activity and, therefore, clinically useless [7-9, 13].

Even though there are already several reviews which describe different classes of EPIs [11, 13-31], most focus on a particular source of compounds or on a class of pumps or bacteria. Herein, it is intended to present a comprehensive review of the several classes of EPIs from natural and synthetic sources and from existing drugs in therapeutics and to highlight structure-activity relationship studies in the referred efflux pumps (ABC, RND, MFS, SMR and MATE), which can be useful for medicinal chemists in the pursuit of novel compounds for this goal.

2. ABC

The ABC transporters are primary transporters, and they exist in every membrane systems, whether the organism is prokaryotic or eukaryotic. P-Glycoprotein (P-gp) is a very well known human ABC transporter, involved in the resistance of cytotoxic drugs used in cancer therapy [32-34], whose modulation has been regarded as a study field [35-37]. Structurally, an ABC transporter consists of four conserved domains, two transmembrane domains, and two cytoplasmic domains, which bind to nucleotides and, therefore, are involved in ATP binding [32, 38]. In fact, the nucleotide binding domains are responsible for hydrolyzing ATP, which will lead to conformational changes in the attached transmembrane domains, making it possible for substrates to cross the lipid bilayer of the membrane, either in an inwards or an outwards fashion [39].

The bacterial transporters are highly selective for sugars, amino acids, metallic cations, organo-iron complexes, vitamins, and antibiotics [32, 40]. They are found in Gram-positive bacteria, such as *Enterococcus faecalis* and *Staphylococcus aureus*, and confer resistance to macrolides and bacitracin. However, only a limited number of ABC pumps are associated with MDR, which means that ABC pumps do not play a pivotal role in conferring antibiotic resistance in bacteria [32-33].

As such, bacterial ABC transporter inhibitors are scarce. The few EPIs discovered in this context are mainly against MsrA (Figure 2, PDB 1NWA), whose tridimensional structure has been elucidated [41].

The structures of the ABC inhibitors that will be further detailed throughout this section are comprised in Figure 3.

2.1. ABC inhibitors

2.1.1. Natural Products

The search for natural products comes from the reason that plants produce antimicrobials to protect themselves from exogenous organisms. It is thought that plants can produce anti-MDR compounds to fight the microbial resistance

phenomenon. Carnosic acid (**1**) was one of the major products isolated from the plant *Rosmarinus officinalis*. When tested against the macrolide resistant strain of *S. aureus*, expressing the MsrA pump, compound 1 displayed favorable results, showing an impressive 8-fold potentiation of erythromycin [42].

An extract of *Lycopus europaeus* led to the isolation of two new isopimarane diterpenes, methyl-1 α -acetoxy-7 α ,14 α -dihydroxy-8,15-isopimarandien-18-oate (**2**) and methyl-1 α ,14 α - diacetoxy-7 α -hydroxy-8,15-isopimarandien-18-oate (**3**), which showed no antibacterial activity, but were able to reduce erythromycin's MIC from 256 μ g/ml to 128 μ g/ml, therefore causing a 2-fold potentiation. This behavior was similar to other isopimarane diterpenes isolated in this study, as well as for 5,9-dihydroxygeranylinalool (**4**) and 9-acetoxy-5-hydroxygeranylinalool (**5**), two diterpenoids [43-44].

Phytol (**6**) is an acyclic isoprenoid, and its EPI activity against the ABC pumps of *E. coli* was assessed in combination with nalidixic acid. Additionally, 15 derivatives were synthesized. One derivative in particular, compound 7, was able to decrease the MIC of nalidixic acid by 16-fold, and four other derivatives reduced the MIC by 8-fold. This effect was caused by the inhibition of ATP-dependent efflux pump and down-regulation of the efflux gene. *In silico* absorption, distribution, metabolism, and excretion (ADME) analysis was performed, taking into account parameters such as solubility in water, human intestinal absorption, plasma protein binding, blood-brain barrier penetration, hepatotoxicity and cytochrome P450 (CYP450) inhibition. Results showed all five active derivatives presented low permeability into the central nervous system and displayed moderate intestinal absorption. In terms of plasma protein binding level, all five compounds exhibited levels below 90%, suggesting favorable distribution of the compounds into the blood. They also did not present hepatotoxicity, unlike reserpine, making them good lead compounds [45].

Ursolic acid (**8**) is a pentacyclic triterpenoid isolated from diverse natural sources. Its wide activity includes anti-inflammatory, antimicrobial, diuretic, and antitumor activity, among others. When tested against *Escherichia coli* strains that were resistant and susceptible to nalidixic acid, ursolic acid did not display intrinsic antibacterial activity. However, ursolic acid (**8**) was able to reduce the MIC of nalidixic acid by 2-fold, when both drugs were used in combination. This synergy led to further research on this scaffold. The first derivative prepared was the 3-*O*-acetyl ursolic acid, and showed a 4-fold decrease in the MIC of nalidixic acid. The lipophilic nature of this derivative, allied with the increased potency, led to the synthesis of other lipophilic ester derivatives. The methyl and ethyl esters of 3-*O*-acetyl ursolic acid did not show any increment in the activity. However, a further increase in the ester carbon chain increased the drug reversal potential by 2-fold, in comparison to 3-*O*-acetyl ursolic acid, against the resistant strain. This is the case of the isopropyl (**9**) and the *n*-butyl (**10**) derivatives. The addition of a pentyl group led to a decrease in activity. Furthermore, ursolic acid (**8**), and derivatives **9** and **10**, reduced the MIC of tetracycline by 2-fold, 8-fold, and 8-fold,

1
2 respectively. When these compounds were tested against
3 ATP-dependent efflux pumps, it was found that derivatives 9
4 and 10 were successful in inhibiting these pumps in terms of
5 the liberated inorganic phosphate. Molecular modeling
6 studies with the YojI pump, using maltose/maltodextrin
7 transport ATP-binding protein as a homology model, showed
8 that 9 was bound to the nucleophilic Ser-78, to the aromatic
9 Phe-81, Trp-85 and Leu-86, the acidic Glu-94 and Glu-144
10 and the basic Ala-79, Ala-139, Ala-140, Ala-141, Ala-143,
11 Arg-74, Arg-122, Arg-146, Leu-107, Val-80, Lys-96, and
12 Ile-123 in the nucleotide binding domain. Derivative 10 was
13 also subjected to the same kind of studies using the same
14 model, and was predicted to bind in the same region [46].

15 A study with flavones and isoflavones from the plant
16 *Sophora moorcroftiana* revealed that diosmetin (11) was
17 able to inhibit three drug resistant strains of *S. aureus*,
18 including the ABC family MsrA overexpressing strain,
19 potentiating the effects of norfloxacin and streptomycin [47].
20 These results corroborate the works of Chan *et al* (2013),
21 who had previously studied the effects of diosmetin (11) as
22 an EPI [48]. This group observed a synergistic effect of this
23 flavonoid with erythromycin, which was 5-fold greater than
24 that of verapamil [48].

25 4-Hydroxy- α -tetralone (12) isolated from the plant
26 *Ammannia multiflora*, showed enhancement against nalidixic
27 acid resistant strains of *Escherichia coli*, prompting the
28 synthesis of derivatives in order to infer their mechanism of
29 action. Five derivatives were synthesized, and it was shown
30 that compound 13 was able to inhibit the expression of the
31 ABC transporter protein gene *yojI*, which encodes the ABC
32 protein YojI, and was successful in combination with
33 tetracycline [49]. Molecular modelling studies with
34 compound 13 and a homology model of the YojI protein
35 revealed the following bound residues: Ser-78
36 (nucleophilic), Leu-86, Ile-123, Ala-137, Ala-140, Ala-141,
37 and Ala-143 (hydrophobic), Glu-144 (acidic), Arg-122
38 (basic), Phe-81 (aromatic), Glu-144 (acidic), Arg-146 and
39 Lys-96 (basic), and Trp-85 (hydrophobic), the same region
40 as noted for ursolic acid derivatives 9 and 10. Compound 13
41 showed good affinity and strong hydrophobic interaction
42 with the YojI protein, corroborating the fact that this
43 compound acts by the inhibition of ATP dependent efflux
44 pumps [49].

45 The plant *Portulaca oleraceae* is used in folk medicine for
46 its anti-inflammatory, anti-septic and diuretic effects, among
47 others. A study of its fatty acids demonstrated that linoleic
48 (14) and oleic acids (15) had synergistic activity with
49 macrolides for the MsrA overexpressing strain of
50 methicillin-resistant *S. aureus* (MRSA). In fact,
51 erythromycin's activity was enhanced when in combination
52 with either of these two compounds, likely through the
53 inhibition of the MsrA ATP dependent pump. Since these
54 fatty acids are essential for humans, low toxicity is expected,
55 making them promising agents to use in combination with
56 antibiotics [50].

56 Lysergol (16) is a clavine alkaloid of the ergoline family.
57 Recently, its EPI activity was studied, and derivatives were
58 synthesized [51]. The compounds were tested against

susceptible and resistant to nalidixic acid strains of *E. coli*
and a tetracycline resistant strain. While not possessing
antibacterial activity, lysergol (16) showed synergy with
nalidixic acid at the concentration of 10 μ g/ml, reducing its
MIC by 8-fold in the resistant strain of *E. coli* and 4-fold in
the susceptible strain. Of the thirteen semi-synthetic
derivatives prepared, derivatives 17 and 18 reduced nalidixic
acid's MIC by 8-fold, twice as much as lysergol (16), in the
susceptible strain. A 4-fold potentiation of nalidixic acid was
observed for the three compounds when tested against the
resistant strain. As for the tetracycline resistant strain, all
three compounds were able to reduce tetracycline's MIC by
8-fold. Further studies were performed, in order to conclude
whether or not these compounds were capable of interfering
with ATP-dependent efflux pumps, and it was proven that all
three compounds inhibited ATPase activity, concerning the
liberation of inorganic phosphate. A down-regulation of the
gene *yojI* was also observed when the tetracycline resistant
strain was treated with lysergol (16) and its two most potent
derivatives, 17 and 18, which further confirms the activity of
these compounds as ABC transporter inhibitors [51].

2.1.2. Existing Drugs

As part of a screening program for P-gp inhibitors,
GG918 (19) was discovered. This synthetic compound had
already showed success in increasing the intracellular
concentration of paclitaxel in cancer cells. In *S. aureus*, this
compound was able to slightly potentiate the activity of
norfloxacin in a strain overexpressing the ABC efflux pump
MsrA, suggesting that it may possess minimal activity over
this pump [52].

The human P-gp inhibitors, vanadates, have been
studied as inhibitors of the MutS pumps, belonging to the
ABC ATPase superfamily. In fact, these compounds have
demonstrated activity against the MutS in *P. aeruginosa* and
E. coli. Decavanadate was the most effective, followed by
orthovanadate (20), and they act non-competitively, possibly
by replacing the inorganic phosphate. The fact that the ABC
ATPases inhibited by orthovanadate are phosphorylated
leads to the assumption that the low inhibitory effect could
be attributed to the absence of a stable phosphorylation state
of MutS [53].

3. MFS

The MFS is the most extensively studied family of
transporters, present in both Gram-positive and Gram-
negative bacteria, and comprises uniporters, symporters and
antiporters. It also constitutes the largest family of secondary
transporters, with over 10 000 sequenced members. Its
substrates are diverse, and can range from ions, to
carbohydrates, lipids, amino acids, nucleosides, among
others. As many members of the MFS originate bacterial
MDR, these transporters can pose as a good system for
modulation studies [7, 19, 38, 54].

With the elucidation of the crystal structure of the MFS
pump EmrD of *E. coli* (Figure 4, PDB 2GFP) [55], a general

structure could be proposed for MFS transporters: twelve transmembrane helices that form a compact structure, with four of these transmembrane helices facing away from the interior cavity and the rest forming the internal cavity, constituted mostly by hydrophobic residues [7].

The efflux of drugs is only possible if the carrier alternates between two conformations, so that the substrate can bind to the convenient site in either side of the membrane. However, a limitation concerning the energy involved to displace the exposed hydrophilic surface of a protein can be inferred. It has been proved that either the oligomerization domain or the transport subunit provide the support needed to the movement of the substrate binding site, through a rigid-body rotation of the substrate binding domain, referred to as the "elevator mechanism" [56-57]. For this family, the most extensively studied efflux pump is the NorA pump of *S. aureus*.

The inhibitors that have proven activity against efflux pumps of the MFS family are gathered in **Figure 5**.

3.1. MFS inhibitors

3.1.1. Natural Products and Derivatives

Terpenes and terpenoids, existent in the essential oils of plants, have showed promising therapeutic potential in various fields. Therefore, studies about EPI potential of these compounds were carried out. Monoterpenes nerol (**21**), estragole (**22**), and dimethyl-octanol (**23**) were tested against *S. aureus*. Compounds **21** and **23** were able to enhance the activity of norfloxacin, decreasing its MIC against the NorA overexpressing strain while not displaying intrinsic antibacterial activity. These open chain terpenes proved to be more effective than **22**, which carried a benzene ring, at inhibiting the NorA pumps [58].

Citronellal (**24**) and citral (**25**) are monoterpenes, and were chosen as starting material for the synthesis of new amide derivatives. The basis for this study was the fact that aromatic amides are recognized as potent EPIs, whereas alkyl, alkenyl, and alkylnil amides had not yet been explored. The compounds obtained were 5,9-dimethyldeca-2,4,8-trienoic acid amides and 5,9-dimethyldeca-2,8-dienoic acid amides, whose activity for the potentiation of ciprofloxacin in *S. aureus* was assessed. Of the first 22 synthesized compounds, seven were able to reduce ciprofloxacin's MIC by 4-fold. Further modifications were performed, and the C-9 methyl group was replaced by an amide, yielding compounds with similar potency as the previous ones. Derivatives subjected to hydrogenation led to decreased activity, which reveals that unsaturation is an important feature for antimicrobial potentiation. The mechanism of inhibition was assessed through ethidium bromide efflux, confirming these compounds act by inhibiting NorA, being compound **26**, a 9-formyl-5-methyldeca-2,4,8-trienoic acid amide, the most potent inhibitor [59].

Ferruginol (**27**), a diterpene, showed efficiency in inhibiting the efflux of ethidium bromide, proving its activity as a NorA inhibitor. It also showed a decrease in norfloxacin

efflux in *S. aureus* [60]. Totarol (**28**), a phenolic diterpene, also showed a reduction in ethidium bromide efflux, suggesting activity against NorA pump, even though its mechanism is not yet known [61].

The triterpene ginsenoside 20(S)-Rh2 (**29**), a trace compound from red ginseng, also enhanced the effect of ciprofloxacin against *S. aureus*, through a NorA-mediated efflux inhibition, promoting the antibiotic accumulation in the bacteria [62].

Reserpine (**30**) is a natural alkaloid derived from *Rauwolfia vomitoria*, known as an anti-hypertensive agent, with inhibitory activity against human P-gp and breast cancer resistance protein (BCRP) [63-64]. Reserpine (**30**) has proven to be an effective inhibitor of the Bmr multidrug transporter in *Bacillus subtilis*, diminishing the efflux of ethidium bromide. Its structural analogue rescinnamine also had EPI activity, in a smaller extension than reserpine (**30**) [65]. Through DNA sequencing of the *bmr* genes of mutants, it was shown that reserpine (**30**) interacts with the residues Phe-143, Val-286, and Phe-306 of Bmr pumps, inhibiting drug transport [19]. Compound **30** has shown potential as an EPI for the inhibition of NorA efflux pumps in *S. aureus*, which are structurally related to Bmr pumps, reversing susceptibility to fluoroquinolones, demonstrating reductions up to 4-fold in MIC for some tested strains. It has been shown that **30**'s effect is more pronounced in hydrophilic fluoroquinolones, such as ciprofloxacin [66]. The mechanism by which **30** performs the inhibitory activity is through direct binding to the transporters that mediate drug efflux, acting as a competitive inhibitor [19, 67]. Reserpine (**30**) also succeeded in inhibiting the chloramphenicol efflux by CmlR1 and CmlR2 of *Streptomyces coelicolor*, the ciprofloxacin and norfloxacin efflux in *Listeria monocytogenes*'s pump Lde, and the kanamycin and fusidic acid efflux by LmrS from a MRSA clinical isolate. On the other hand, for *L. monocytogenes*, **30** was not able to reverse susceptibility to linezolid and lincomycin, which suggests that **30** does not completely overlap with the structure binding sites of efflux pumps, being the inhibition dependent on the type and nature of the substrate [19]. However, reserpine (**30**) shows neurotoxicity at the concentration required to inhibit NorA, leading to the need to investigate new inhibitors [67].

Piperine (**31**), an alkaloid found in the fruits of *Piper nigrum*, was found to be an inhibitor of ciprofloxacin efflux in *S. aureus*, reducing the MIC by 2-fold. It is thought that compound **31** acts as a direct inhibitor of drug efflux. Compound **31** inhibits drug transport of the pump MdeA, potentiating the effects of benzalkonium chloride, doxorubicin, daunorubicin, novobiocin, tetraphenylphosphonium chloride, rhodamine 6G, virginiamycin and mupirocin [17, 19-20]; it also inhibits ciprofloxacin efflux by NorA [68].

Given these positive results obtained with piperine (**31**), further research into this scaffold was performed. Kumar *et al.* (2008) performed a screening of about 200 structurally diverse compounds for the inhibition of NorA efflux pump, choosing ethidium bromide as a substrate, since the only

mechanism through which this compound is expelled is active transport (68). About 50 compounds were able to increase the intracellular concentration of ethidium bromide by 4-fold, while not displaying antibacterial activity. Five of these compounds were more potent than piperine (**31**), being three of these compounds selected for further studies: **32**, **33**, and **34**, being **32** and **34** more potent than **31** by 40-fold [18, 68]. Assumptions regarding structure-activity relationship (SAR) studies of these molecules could be drawn (Figure 6). It was demonstrated that the introduction of an alkyl group at C-4 position, mainly an isopropyl, isobutyl or di-isopropyl group, coupled with the substitution of the piperidinyl moiety with an aromatic amine, such as aniline, would lead to maximum potentiation of ciprofloxacin; if the anilinyll moiety is substituted with a 2-hydroxymethyl group or a nitrile, regardless of the positional isomery, ciprofloxacin activity would increase. Replacing the piperidinyl moiety with a phenylacetamide yields good potentiation of ciprofloxacin, reducing its MIC by 4-fold, regardless of the isomery. Unsaturation is crucial for the potentiation, as well as the amide. Lastly, the retention of 3,4-methylenedioxyphenyl or 4-methoxyphenyl groups in the 1,3-benzodiol moiety also gives favorable results [68-70].

Plants of the *Berberis* species have the ability to produce the alkaloid berberine, which is a plant secondary metabolite produced in response to microbial invasion, whose activity is enhanced by a flavolignane also produced by these species, 5'-methoxyhydronecarpin (**35**) [71]. Compound **35** exists in a mixture of anion and neutral compound at physiological pH, since it has a phenolic group with a pKa of 7.3. Due to the acidic properties of this 7-OH group, **35** is likely to be a specific microbial MDR inhibitor, as flavonoids with P-gp inhibitor properties usually possess alkylated 7-O groups. In fact, when added to a wild type strain of *S. aureus*, compound **35** caused a decrease in norfloxacin's MIC to 0.25 µg/ml. Furthermore, when tested with berberine and palmatine, **35** appeared to completely inhibit NorA, suggesting it acts as a non-competitive inhibitor [71].

A number of hydronecarpin-type flavolignanes were synthesized in order to perform SAR studies. Flavolignanes with and without phenolic groups at the 5 and 7 positions had comparable activity. Diosmetin (**11**), the parent compound, presents hydroxyl groups in the A ring and was the most potent compound. Peracetate derivatives of **35** have approximately the same potency as their parent compounds, and could be considered prodrugs, if deacetylated by *S. aureus*, which seems unlikely. Considering the D ring, compounds bearing a 3''-methoxy-4''-hydroxy moiety displayed good activity, and 3'',5''-dimethoxy-4''-hydroxy moieties conferred a slightly less active compound. As for the B ring, the presence of an H or an OH is not critical for activity [72]. In addition, a flavone isolated from *Alkanna orientalis*, sarothrin (**36**), blocked ethidium bromide efflux by *S. aureus*, suggesting its potential activity as a NorA inhibitor [73].

Biochanin A (**37**), an isoflavone, also showed potentiation of norfloxacin and berberine against wild-type *S. aureus* and *Bacillus megaterium*. An analogue of luteolin, orobol (**38**),

was quite active as a NorA inhibitor, unlike luteolin itself. It was shown that flavones which have a single methoxy in its B-ring are more potent than flavones with two methoxy groups in the B-ring [74].

Another flavone, baicalein (**39**), was capable of potentiating the effect of ciprofloxacin in *S. aureus*. Studies aimed at measuring the efflux of ciprofloxacin and pefloxacin, a poor NorA substrate, were performed, and showed that compound **39** was much more potent at restoring ciprofloxacin sensitivity than pefloxacin, leading to the conclusion that this flavone acts as a NorA efflux inhibitor, even though its mechanism remains unknown [75].

Maia *et al.* (2011) identified six flavonoids from the plant *Praxelis clematidea*: apigenine, genkwanin, 7,4'-dimethylapigenin, trimethylapigenin, cirsmaritin, and tetramethylscutellarein (**40**) [76]. These methoxylated flavones showed no antibacterial activity, but were able to reduce the MIC of norfloxacin at least by 2-fold and up to 16-fold, as was the case of **40**, at a concentration of 8 µg/ml, being therefore considered NorA efflux pump modulators. It is thought that the lipophilicity of the flavonoids, strengthened by the presence of methoxyl groups, is responsible for this activity [21]. Presence of a methoxy in the 4' position proved to be decisive for activity, as flavonoids containing this group were more potent. The number of methoxy groups is also essential for potency, being the most methoxylated compounds the most active [76]. Tiliroside (**41**), an amphipathic kaempferol glycoside, reduced the concentration of antibiotic needed to inhibit the growth of bacteria. The lipophilicity of the flavone moiety may be a determinant factor for the activity displayed. Tiliroside (**41**) showed no antibacterial activity against *S. aureus*, but showed a decrease in the MIC of fluoroquinolones up to 16-fold, when at a concentration of 64 µg/ml. The reduction in the MIC of biocides, such as ethidium bromide and benzalkonium chloride, was impressive, with the MIC of acriflavine showing a decrease by 128-fold at the concentration of 64 µg/ml and 32 µg/ml. These results suggest that **41** might additionally act on pumps other than NorA [77].

Kaempferol 3-O- α -L-(2, 4-bis-*E-p*-coumaroyl)rhamnoside (**42**) was also able to potently inhibit in a concentration dependent manner NorA-mediated ethidium bromide efflux in *S. aureus*. This compound also increased the antibiotic effect of norfloxacin against a wild-type and a NorA overexpressing strain of *S. aureus*. However, it has to be considered that **41** and **42** are flavonoid glycosides and cinnamoyl esters, and that their activity can become compromised with the activity small intestine β -glucosidases or plasma esterases, even though the activity might possibly lie in the kaempferol moiety, with **41** and **42** acting as prodrugs. The authors hypothesized that if the activity of compound **42** is compromised, this compound could be used as adjuvant of ciprofloxacin for topical infections [78].

Since **42** showed promising results as a NorA EPI, the same group performed a screening of a library of 117 chalcones, due to their structural similarities with the coumaroyl substituents [79]. Ten out of the 117 screened chalcones

presented medium efflux inhibition at 20 µg/ml. The most potent chalcones, such as **43**, bear a dimethylaminoethoxy moiety, which might disrupt the proton gradient, thus eliminating the energy source of NorA. Five of these chalcones possessed a hydroxyl group at the 4'-position, and potentiated the activity of berberine against *S. aureus* and *B. cereus*, suggesting that this substituent is also important for activity. The compound with a methoxy group at position C-2 has shown to present the best activity. The majority of chalcones tested showed a 4-fold reduction of ciprofloxacin's MIC in *S. aureus*, suggesting the presence of a NorA efflux pump inhibitory activity. Compound **43** showed 8-fold and 16-fold reduction of the MIC of ciprofloxacin at 6.25 and 12.5 µg/ml, respectively. However, it also showed antibacterial activity at 25 µg/ml. Some chalcones also presented antiparasitic activity, namely against *Leishmania major* and *Plasmodium yoelii* [79].

Another chalcone, 4',6'-dihydroxy-3',5'-dimethyl-2'-methoxychalcone (**44**), showed good activity as a MDR, causing complete growth inhibition at a very low concentration (3.3 µg/ml) when combined with a sub inhibitory concentration of berberine. When tested in combination with berberine, erythromycin, and tetracycline against strains of *S. aureus*, there was an increase in the activity of all tested compounds in the wild-type *S. aureus*, indicating a mode of action likely related to the NorA pump. This compound was also tested against *B. cereus*, potentiating the effects of the tested antibiotics as well, and displaying a favorable effect, particularly in the case of berberine, where a 30-fold increase in the activity was observed [80].

Pheophorbide A (**45**) is an intermediate of the natural breakdown of chlorophyll. Concentrations as low as 0.5 µg/ml of pheophorbide A with sub-inhibitory concentrations of berberine were enough to completely inhibit *S. aureus* growth. When extended to fluoroquinolones, this compound also proved efficient in potentiating the effect of norfloxacin by 4-fold in a wild-type strain, being its action directed towards NorA [81].

Orizabins, oligosaccharides from resin glycosides, were evaluated for their efflux pump inhibitory activity on strains of *S. aureus*. These amphipathic compounds showed synergy with norfloxacin, whilst showing no antibacterial activity by themselves. Orizabin IX (**46**) completely inhibited the growth of a NorA overexpressing strain at 1 µg/ml, while orizabin XIX (**47**) reversed norfloxacin resistance by 4-fold at 25 µg/ml for the same strain [82]. Murucoidins, which are also resin glycosides, strongly potentiated the action of norfloxacin against a *S. aureus* NorA overexpressing strain by 4-fold at concentrations ranging from 5 to 25 µg/ml. Stoloniferin I potentiated the same activity by 8-fold at a concentration of 5 µg/ml [83].

A study aiming to find new EPIs from the plant *Geranium caespitosum* led to the isolation of novel acylated neohesperidosides. It was found that two compounds, compound **48** and **49**, were successful at restoring susceptibility to berberine, norfloxacin, and ciprofloxacin at

sub inhibitory concentrations, possibly due to NorA inhibition [84].

Capsaicin (**50**), the major constituent of the fruits of the *Capsicum* genus, is a known P-gp inhibitor. Studies were performed, in order to evaluate if its inhibitory activity was also applied to MDR pumps in bacteria. In a strain of *S. aureus* overexpressing the NorA efflux pump, and therefore resistant to fluoroquinolones, **50** proved efficient in increasing the susceptibility to ciprofloxacin, while reducing the emergence of ciprofloxacin-resistant mutants. Molecular modelling studies of the complex capsaicin (**50**)-NorA, using the glycerol-3-phosphate transporter to predict the structure of NorA, showed that compound **50** aliphatic chain extended into NorA's hydrophobic cleft. Furthermore, a weak hydrogen bond between Arg-98 and the aryl hydroxyl contributed to the stability of the complex [85].

Studies in the aqueous extract of *Artemisia absinthium* showed promise in the inhibition of MDR efflux pumps. It was proven that this activity came from caffeoylquinic acids. At a concentration of 20 µM, 4'-5'-*O*-dicaffeoylquinic acid (**51**) potentiated the activity of berberine by 16-fold and of norfloxacin, by 10-fold against a wild-type strain of *S. aureus*, while not displaying effects in a *norA*-deleted strain, confirming this compound's NorA inhibition ability [86].

Olympicin A (**52**) is an acylphloroglucinol, found in the aerial parts of *Hypericum olympicum*. This molecule had some efflux impeding ability, proven by the improvement of intracellular accumulation of enoxacin in a NorA overexpressing strain of *S. aureus*, at 50 µg/ml [87].

Riparins are natural alkylamides found in the plant *Aniba riparia*. Since the extraction of these compounds comes with very low yields, synthetic approaches were developed. As such, the fundamental core of riparins, Rip-A (**53**), was synthesized and derivatives were prepared, Rip-B (**54**) to E (**55**). Rip-E (**55**) showed good growth inhibitory activity against *S. aureus*, attributed to their lipophilic nature and the presence of hydroxyl groups at the benzamide moiety. Oppositely, Rip-B (**54**), with two methoxy groups at the phenyl-ethyl moiety did not present antibacterial activity, presenting instead a decrease in the MIC of ciprofloxacin and norfloxacin, similarly to reserpine. Combined with the ability of decreasing ethidium bromide's MIC, the activity of compound **54** was attributed to NorA inhibition, suggesting its potential as an EPI [88].

Extracts of *Mirabilis jalapa* have shown good results in reversing fluoroquinolone resistance in strains of *S. aureus* overexpressing the NorA efflux pump. One compound in particular, *N-trans*-feruloyl-4'-*O*-methyl-dopamine (**56**), caused an 8-fold reduction in the MIC of norfloxacin, making it a potential lead in the search for structurally similar synthetic alkylamines [89].

Synthesis of derivatives was undertaken, taking *N-trans*-feruloyl-4'-*O*-methyl-dopamine (**56**) as a model. The aim was to identify active compounds from natural sources and, as such, couplings between cinnamic acid derivatives and natural-occurring amines were chosen. SAR criteria was taken into account, as it is known that substitution of the aromatic rings, methoxy or hydroxyl substitution, double

bonds and aromatic ring nature in the amine part influence the activity. The lead compound was *N-trans*-3,4-*O*-dimethylcaffeoyl dopamine (**57**) since it showed the same activity as the natural compound. It was found that, for the cinnamic moiety, a hydroxyl substitution on the aromatic ring appears to be better than a methoxy group or unsubstituted derivatives and the double bond is essential for activity. As for the amine part, trisubstitution on the aromatic ring increases antibacterial activity, at the cost of decreased efflux pump inhibition. Methoxy substitution yields compounds with better results than hydroxyl substitution, which was better than no substitution and tryptamine combinations showed the best results (Figure 7). Among the compounds tested, compound **58** showed potentiation of norfloxacin comparable to that of reserpine (**30**). At a concentration of 30 μ M, **58** showed good results for the inhibition of ethidium bromide efflux. For these compounds, it was shown that the activity reaches its highpoint when the phenyl ring is substituted with two hydroxyls [89].

Grapefruit oil fractionation led to the isolation of three compounds, two coumarin derivatives and one bergamottin derivative. The bergamottin epoxide (**59**) showed a reduction of ethidium bromide's MIC by 6-fold on different MRSA strains, and a 20-fold reduction in norfloxacin's MIC. This reduction was also achieved when the coumarin epoxide (**60**) was present, and suggests an activity towards NorA efflux pumps [90].

Coumarins isolated from *Mesua ferrea* were also studied for their efflux pump inhibitor activity against clinical strains and NorA overexpressing strains of *S. aureus*. Seven coumarins were tested, and showed good results in inhibiting ethidium bromide efflux. Two compounds, **61** and **62**, showed potential EPI activity against the NorA overexpressing strain and a MRSA strain, using norfloxacin as a substrate, which makes them desirable compounds for the synthesis of derivatives [91].

Screening of natural products with P-gp inhibitory effects (92, 93) led to the identification of two compounds with NorA inhibitory activity, osthol (**63**) and curcumin (**64**) [36, 92-93]. Curcumin (**64**), derived from the rhizomes of *Curcuma longa*, has additionally already shown activity as an anti-inflammatory, antioxidant, antiviral, and anticancer agent [36, 92-93]. These compounds were effective in reducing the MIC of ciprofloxacin by 4- and 8-fold, respectively [94].

A penta-substituted pyridine, 2,6-dimethyl-4-phenylpyridine-3,5-dicarboxylic acid diethyl ester (**65**), was isolated from the rhizomes of *Jatropha elliptica*. This compound, when in association with ciprofloxacin, showed a strong effect against NorA efflux pumps in *S. aureus* [95].

A methanol extract of the plant *Dalea spinosa* yielded the isolation of six compounds, whose EPI potential was analyzed against wild-type *S. aureus* and mutant strains. It was shown that spinosan A (**66**), at a concentration of 48 μ g/ml, and its acetate **67**, at a concentration 42 μ g/ml, were able to decrease berberine's MIC by 8- and 62-fold, respectively, against the wild-type. Other compounds were also able to reduce berberine's MIC, but to a lesser extent.

Compound **67** also managed to decrease berberine's MIC in the NorA overexpressing strain, suggesting inhibitory activity in this efflux pump [96].

3.1.2. Synthetic Compounds

The INF series (compounds **68-72**) resulted from an initial screening of 9600 structurally diverse compounds, in order to find NorA inhibitors for *B. subtilis*, not expressing Bmr. The NorA substrate chosen was ethidium bromide [67].

Since this screening did not focus on a specific chemical group, the most active compounds were divided into several groups. Several indole derivatives were active, which did not come as a surprise, since reserpine (**30**) itself has an indole moiety. Another large group was the trichloromethylamine containing compounds, which were not further explored as their likelihood to be toxic was high. Biphenyl urea derivatives were also active, and other compounds, with no obvious similarities, showed activity as well. Five compounds were selected for further tests, INF 55 (**68**), INF 240 (**69**), INF 271 (**70**), INF 277 (**71**) and INF 392 (**72**). These compounds were active at concentrations of 5 μ g/ml or less, and included the most potent compound, **72**, the most potent indole, **68**, and the most potent biphenyl urea, **71**. Derivative **72** was able to reduce resistance to ethidium bromide and ciprofloxacin by 8-fold at a concentration of 0.4 μ g/ml. All five inhibitors were more potent than reserpine (**30**) at promoting the activity of ciprofloxacin in *S. aureus* and, by being structurally different, it was hypothesized that a potent, nontoxic lead would be identified. These compounds also proved effective in inhibiting the Bmr multidrug transporter in *B. subtilis* and two compounds, **68** and **71**, also enhance fluoroquinolone activity in *Streptococcus pneumoniae* [67].

The results obtained with INF 55 (**68**) in *S. aureus* as an inhibitor of the NorA efflux pump led to an increased interest in this kind of scaffold. SAR studies regarding the C-5 proved that substitution in this carbon is crucial for activity. Substitution with a nitrile group leads to retention of potency, and carbonyl based electron-withdrawing groups at C-5 would result in molecules with no activity [97]. Three dimensional-quantitative structure-activity relationship (3D-QSAR) studies predicted that 2-aryl indole derivatives would be NorA inhibitors. Functionalized INF 55 (**68**) showed a decreased MIC for berberine, a MDR transporter substrate. Berberine is an amphipathic cation alkaloid. From the series of 2-aryl-5-nitroindoles synthesized by Samosorn *et al.* (2009), the alcohol **73** was the most effective in inhibiting berberine efflux, as well as other antibiotics, such as ciprofloxacin, in *S. aureus* [98]. Later, the same group developed a strategy that consisted in a molecule presenting dual action: an antibiotic and a MDR inhibitor. Therefore, they combined berberine, a hydrophobic cation that is a MDR substrate, with INF 55 (**68**), a MFS inhibitor. These efforts yielded compound **74** [17-18, 99]. In terms of antibacterial activity in *S. aureus*, the hybrid was about 100 times more active than berberine alone, and the difference was even higher when tested against a NorA overexpressing

1
2 strain of *S. aureus*. *Enterococcus faecalis* also presented
3 resistance to berberine, but was susceptible to **74**. [99]
4 Synthesis of sulfur derivatives was achieved based on the
5 premise that the activity of **68** is less dependent on the nitro
6 group than the 2-arylindole. Benzothiophene, thiophene, and
7 benzofuran derivatives, such as compounds **75**, **76**, and **77**,
8 respectively, were able to synergize with ciprofloxacin and
9 ethidium bromide, increasing their intracellular
10 concentration and leading to the conclusion that these
11 compounds inhibit NorA in *S. aureus*. [100]

12 Taking into account the fact that the indole NH is not
13 essential for efflux pump inhibition, Samosorn *et al.* (2009)
14 simplified the indole moiety of berberine-based hybrids,
15 along with the effect of a methylene ether linking group.
16 Their results showed that a hybrid molecule with oxygen in
17 the linkage chain had stronger antibacterial and MDR pump
18 inhibitory activity than the original **74** [101]. On the other
19 hand, removal of the indole moiety produced compounds
20 with lower dual activity, and this structural simplification led
21 to compounds with low antibacterial activity, but with
22 retained NorA pump inhibitory activity. These data suggest
23 that while the indole moiety is not necessary for NorA pump
24 inhibition, it is required for the antibacterial activity of
25 hybrid compounds [101].

26 A recent study identified a novel class of NorA EPIs with a
27 polysubstituted indole moiety. Twenty molecules were
28 highly active in inhibiting ethidium bromide efflux in *S.*
29 *aureus*. However, the best results were obtained with
30 compounds **78** and **79**, which were derivatives that presented
31 three and four carbon atom chains, respectively. Compound
32 **78** presented the best half maximal inhibitory concentration
33 (IC₅₀), with a concentration of 1.8 μM. Compound **79**
34 exhibited similar IC₅₀ (2 μM) and ethidium bromide
35 inhibition as **78**. Compounds with shorter or longer carbon
36 chains did not present such good results, leading to the
37 conclusion that three or four carbons make up the right
38 distance between the indole scaffold and the basic center,
39 thus presenting the best inhibitory effect. When tested for
40 their synergy with ciprofloxacin, both **78** and **79** were able
41 to restore the antibiotic's activity [102]. It was found that the
42 substitution of the C-5 in the indole results in a potent EPI,
43 particularly if there is a propoxyl chain carrying terminal
44 cyclic amino groups, which appears to be crucial for NorA
45 inhibition at low concentrations. The presence of the *N*-
46 benzyl moiety preserves inhibition and contributes to the
47 modulation of the biological effects and ADME properties,
48 depending on its substituents [103].

49 Another successful example of a hybrid antibacterial/EPI is
50 the fluoroquinolone scaffold accommodating a bis-aryl urea
51 EPI moiety at the C-7 position. This kind of compounds was
52 successful in inhibiting NorA pumps in *S. aureus*.
53 Compound **80**, which incorporates a bis-aryl urea motif into
54 the ofloxacin core, proved to be a potent inhibitor of MFS
55 efflux pump systems, achieving 84 % inhibition rate at 10
56 μM. Moreover, these compounds also successfully inhibit
57 MepA pump systems from the MATE family [17-18, 104].
58 Fluoroquinolones containing a thiopyranopyridine moiety at
59 the C-7 position were synthesized and their activity was

60 tested against strains of *S. aureus*. However, these
derivatives did not seem to be NorA substrates. Further
investigations into thiopyranopyridinylquinolone esters were
carried out. Some of the synthesized compounds displayed
better inhibitory activity than reserpine (**30**), namely
quinolone esters **81**, **82** and **83**, and showed significant MIC
reduction of ciprofloxacin. Compounds **82** and **83** also
showed a 32-fold MIC reduction of ciprofloxacin for strains
of *S. aureus* overexpressing MepA pumps from the MATE
family [18, 105]. In terms of established SAR, these
compounds definitely do not need the fluorine atom at the C-
6 position for the antibacterial activity of
thiopyridinylquinolones. A methyl at the C-8 position yields
a compound with enhanced activity against Gram-positive
bacteria for 6-amino derivatives. The thiopyranopyridine
moiety at the C-7 position is crucial for NorA and MepA
inhibition (**Figure 8**) [105].

Derivatives of 2-(4-propoxyphenyl)quinolone were
synthesized based on the premise that its large hydrophobic
area and ability to establish an electrostatic interaction would
make them suitable inhibitors of NorA. These derivatives
also mimic the quinolone antibacterial core and possess a
versatile scaffold, making it prone to be very simply
chemically modified. These classes of compounds were
obtained by performing modifications in the 2-phenyl-4*H*-
chromen-4-one moiety, which is a common feature of
flavone and flavolignane EPIs [106].

Studies showed that the *O*-substituted 2-phenyl-4-
hydroxyquinoline derivatives displayed the best activity as
NorA inhibitors. Compounds **84** and **85** showed synergistic
activity against a strain of *S. aureus* overexpressing NorA
efflux pumps comparable to reserpine (**30**) and, comparing
the results obtained with a mutant strain, absent of NorA, it
was found that these compounds are able to completely
restore the antibacterial activity of ciprofloxacin in the
resistant strain [18, 106]. Preliminary SAR studies for this
new class of EPIs showed that the best activity is when the
quinolone core is replaced with the 2-phenyl-4-
hydroxyquinoline group with an alkylation in the C-4
hydroxyl group. 2-Ethylamino chains inserted at the N-1
position of the quinolone nucleus or at the C-4 hydroxyl of
the quinoline moiety provided compounds with better
activity than those carrying the same chain at the C-2 of the
phenyl ring. The best substituent for the C-2 phenyl ring
appears to be the C-4' propoxy group (**Figure 9**) [106].

Taking these results into account, efforts have been placed to
obtain more potent NorA inhibitors, introducing *O*-alkyl or
different *O*-alkylamino chains at the C-4 position. The
strategy was to determine the essential 3D structural
requirements for the inhibition of NorA, based on the NorA
EPIs described in literature through the use of computational
methods, since the 3D structure of this pump was not
elucidated yet. The results led to the building of a possible
pharmacophore, which was used to design novel compounds,
with different substituents at the C-4 position, which were
synthesized and biologically evaluated [107]. The
compounds were assayed for their ability to reduce the MIC
of ciprofloxacin in *S. aureus* strains, and all compounds

1 displayed this decrease for the strain overexpressing NorA
2 pumps. One compound in particular, **86**, showed a 16-fold
3 reduction in this strain for ciprofloxacin's MIC. SAR studies
4 for this emerging class show that the best activities come
5 from the compounds bearing the 2-ethylaminoalkyl chains
6 linked to the C-4 hydroxyl group, such as compound **87**. The
7 inclusion of an aliphatic ring including the side chain
8 nitrogen atom led to retention of activity, whereas inclusion
9 in an aromatic ring led to loss of activity, where the nitrogen
10 nucleophilicity was reduced. The mono-substituted
11 compounds, like **88**, with a benzyl group were well tolerated,
12 and showed higher ethidium bromide inhibitory activity than
13 compounds with two benzyl groups as substituents (**Figure**
14 **9**) [107].

15 3-(Dihydronaphthyl)-propenoic acid amines were evaluated
16 for their EPI activity. Five compounds were synthesized, and
17 two of them showed good results in inhibiting ciprofloxacin
18 efflux in strains of wild-type and NorA overexpressing *S.*
19 *aureus*. Further studies were performed, given the low EPI
20 activity of this kind of compounds. Fifteen different
21 compounds were synthesized, taking α -tetralone (**12**) as
22 starting material. Out of these, four compounds showed
23 significant MIC reductions for ciprofloxacin for the NorA
24 overexpressing strain. One compound, **89**, even displayed a
25 16-fold reduction, while other remained in the range of 4- to
26 8-fold. These studies allowed SAR to be established.
27 Addition of methoxy or allyloxy substituents at the 6,7-
28 position of the 3,4-dihydronaphthalene leads to lower or no
29 activity, whereas no substitution originated better EPIs.
30 Saturation of the double bonds lowered the potentiating
31 activity (about 2-fold), while unsaturated derivatives made
32 much more potent compounds [108].

33 Boronic species have been described for a variety of
34 activities, namely antimicrobial and antineoplastic.
35 Organoboron compounds, oxazaborolidines, boronic esters,
36 and boronic acids are of particular interest, within this class.
37 Fontaine *et al.* (2014) developed studies on these molecules,
38 starting from a screening of a library of approximately 150
39 compounds, out of which 24 were hit compounds
40 (exemplified with compounds **90** and **91**), and were able to
41 restore the activity of ciprofloxacin by 4-fold at
42 concentrations of 0.5 to 8 $\mu\text{g/ml}$ against the NorA
43 overexpressing strain of *S. aureus* [109]. Preliminary SAR
44 studies demonstrated the need of the boron atom for activity.
45 Most pyridine-3-boronic acids showed best potentiating
46 activity (e.g., compound **90**), whereas pyridine-4-boronic
47 derivatives showed no activity, with exceptions, including a
48 fluorinated ester. The shift of the boronic moiety from C-3 to
49 C-4 led to a loss of activity and the trigonal boronic acid
50 function gave better results than the sodium boronate salts
51 and the potassium trifluoroborate. Concerning the pyridine-
52 3-boronic acids, the most promising compounds appear to be
53 the 6- and the 5,6-disubstituted ones, being the latter
54 compounds less potent. Cyclisation at the C-5 and C-6
55 positions was well tolerated, as was the introduction of a
56 methyl at the C-5 position. For the benzene analogues, which
57 displayed good activity, cyclisation at the C-3 or C-4 was
58 also well tolerated, as was the introduction of a methyl. A

substituent at the *para* position to the boronic moiety proved
to be very important. Two compounds were particularly
efficient in inhibiting NorA, and did not display intrinsic
antibacterial activity and cytotoxicity: the 6-
benzyloxy pyridine-3-boronic acid **90**, which displayed the
highest activity, potentiating ciprofloxacin by 4-fold at a
concentration of 16 $\mu\text{g/ml}$, and the 4-benzylxybenzene
boronic acid **91** [109].

Compound **90**, being the most promising compound, was
considered a hit, and its structure was used as a model for the
synthesis of new derivatives. Therefore, the 6-benzyloxy
group was substituted with (aryl)alkoxy chains with variable
lengths (compounds **92** and **93** as examples), in an attempt to
explore the putative hydrophobic binding site of NorA, and
substituents were added to the phenyl ring. The ether linkage
between the pyridine ring and the 6-benzyloxy group was
substituted as well. The first substitution made was the
replacement of the 6-benzyloxy group with alkoxy chains of
diverse lengths, as to explore the size of the hydrophobic
pocket of NorA. The potency of the compounds increased
with the increase of the size of the side chain, although the
compound with a 11-carbon side chain displayed no activity
whatsoever. A new series of compounds with a phenyl ring
at the end of the side chain were synthesized, with the aim of
enhancing hydrophobicity, and the results showed these
compounds were more efficient. It was concluded that the
compound with the best performance possessed a side chain
with seven carbon atoms and a phenyl group: compounds **92**,
6-(3-phenylpropoxy)pyridine-3-boronic acid, and **93**, 6-(4-
phenylbutoxy)pyridine-3-boronic acid, showed the highest
activity in the potentiation of ciprofloxacin (4-fold). The
introduction of substituents in the phenyl ring of compound
90 led to unsatisfactory results, with the best compound
displaying moderate antibacterial activity. A methyl group in
the C-5 position did not show influence on the activity, and
the modification of the ether linkage led to varied effects:
introduction of a sulfanyl, thus conserving the H-bonding
acceptor capacity, led to similar potentiating activity,
whereas the introduction of an amine linkage, an H-bond
donor, led to a decrease in the activity. Finally, the shift of
the benzyloxy group from the C-6 to the C-5 resulted in no
activity [110].

Compounds AE-848/42434549 (**94**) and AN-465/42885978
(**95**) were hits in a virtual screening of novel NorA efflux
pump inhibitors in *S. aureus*. The structures deemed as
active *in silico* were later synthesized and evaluated on their
ability to prevent ethidium bromide efflux, as well as
ciprofloxacin potentiation. Compound **94** and **95** were as
potent as reserpine (**30**) for ethidium bromide efflux and
more potent at synergizing with ciprofloxacin. These hit
compounds **94** and **95** paved way for the synthesis of new
analogues, which allowed SAR studies to be made, in order
to assess which parts of their structure were determinant for
the inhibition of NorA. For compound **94**, it was shown that
limited modifications to the sulfone and amide group do not
cause significant changes in activity. On the other hand,
modifications to the imidazole ring result in a reduction of
activity. Replacement of the 1-methylpyrrole ring with a

furan also shows a decrease in the activity of the compound. As for compound **95**, the *O*-benzyl group is essential and the introduction of a benzyl in the secondary nitrogen leads to a complete loss of activity. Furthermore, the methyl group of the 2-(1-phenyl-1-propanol) moiety can be replaced with a 2-(1-phenyl-1-ethanol) portion without a decrease in the activity [111].

3.1.3. Existing Drugs

Several drugs already available for the treatment of various diseases have shown potential to be used as MFS EPIs. Reserpine (**30**), mentioned before, is one of them. Other drugs include verapamil, omeprazole, paroxetine and chlorpromazine. Even though bacterial MFS transporters and mammalian ABC efflux systems, as P-gp, lack structural homology, they share similar substrate profiles, which means that mammalian MDR inhibitors can also present bacterial efflux inhibition. However, the concentrations needed for efflux pump inhibition are too high, which means that toxic effects can arise if these compounds are used for this purpose [18], [112]. In fact, the addition of verapamil to chemotherapy was accompanied with cardiac arrhythmia and hypotension [113]. This leads to the requirement of developing analogues of these existing drugs maintaining the desired EPI activity at concentrations that do not display toxicity [112].

The antiarrhythmic verapamil, a known P-gp inhibitor, has shown moderate NorA inhibitory activity in both *B. subtilis* and *S. aureus*, enhancing fluoroquinolone activity [114-115]. However, the best results were obtained with proton pump inhibitors omeprazole (**96**) and lansoprazole. Therefore, a series of pyrrolo[1,2-*a*]quinoxaline derivatives mimicking the omeprazole structure (**96**) were designed, with the aim of evaluating their EPI activity against NorA. All the eleven derivatives showed a reduction in norfloxacin's MIC, being compound **97** the most active, reducing the MIC by 16-fold at 128 µg/ml. These compounds did not show disturbance of the electrical potential and the transmembrane pH, excluding the alteration of the proton-dependent pump as the mechanism of action. Instead, it is thought that these compounds interact directly with NorA. It was shown that the benzimidazole moiety is critical for the activity of these compounds (**Figure 10**), and replacing it with an imidazole, a pyridine or a pyrrolo[1,2-*a*]quinoxaline ring resulted in loss of potency. Furthermore, if there is a methoxy-substituent in the benzimidazole ring, potency increases. Introduction of chlorine in the C-7 of the pyrroloquinoxaline heterocycle provided an efficient restoration of norfloxacin's bactericidal activity. Finally, the benzimidazolyl group was also important for activity [112].

This was not the first time that pyrrolo[1,2-*a*]quinoxaline derivatives were described as EPIs. In fact, a previous study by the same group produced 12 new 4-[116]pyrrolo[1,2-*a*]quinoxalines and one pyrrolo[1,2-*a*]thieno[3,2-*e*]pyrazine compound. Two compounds, **98** and **99**, were more active than reserpine (**30**), diminishing norfloxacin's MIC by 16-fold in a NorA overexpressing strain of *S. aureus*. This study

showed that 4-[116]pyrrolo[1,2-*a*]quinoxalines and their 7-methoxy analogues were more active, in contrast with the chlorine- and 8-phenyl-substituted compounds, that presented the lowest effects. Replacement of the *N,N*-diethylamino group by pyrrolidine, its isomer with restricted conformation, led to enhanced EPI activity, whereas replacing it with a piperidine ring led to more flexible and, consequently, less active compounds. The pyrrolothienopyrazine **99** moiety yielded the best results, suggesting that sulfur, an electron-rich atom, potentiates the EPI activity [116].

GG918 (**19**) was discovered in a screening program aimed to identify inhibitors of mammalian P-gp, and was able to increase the concentrations of paclitaxel. This compound did not present antibacterial activity against the strains of *S. aureus* tested. However, the co-administration with norfloxacin resulted in a 4-fold reduction in the MIC of the NorA overexpressing strain [52].

Two mammalian MDR inhibitors, biricodar (**100**) and timcodar (**101**), confer increased drug sensitivity to cells expressing both the P-gp and multidrug resistance protein (MRP)-1 efflux systems. They also show potentiation of multiple antibiotics in Gram-positive bacteria. In fact, both compounds were able to potentiate the activity of ethidium bromide against *S. aureus*, *E. faecalis*, and *S. pneumoniae*. For the specific case of *S. aureus*, both compounds were able to partially reverse fluoroquinolone resistance. The fact that the MIC of ethidium bromide was non-saturable for the three bacteria tested suggests that **100** targets multiple efflux pumps [117].

A series of polysubstituted pyrroles was screened for their P-gp inhibition activity, which made way for the identification of a lead, and the compounds were further investigated for their bacterial efflux pump inhibition activity. A simplified pyrrole alkaloid, **102**, displayed dual activity as both a P-gp and a NorA inhibitor against *S. aureus*. This compound led to 4-fold improvement in the MIC of ethidium bromide in the NorA overexpressing strain. Compound **102** was also able to reduce ciprofloxacin's MIC by 8-fold when at 50 µM [118].

Tariquidar (**103**), a third generation MDR modulator, with activity in inhibiting P-gp and BCRP efflux systems, has shown promise in inhibiting efflux pumps in *S. aureus*. In fact, this compound showed an increase of the isotopic form of ciprofloxacin in all investigated strains, displaying best results in the strain that overexpresses the NorA pump. [119] The class of phenylpiperidine selective serotonin reuptake inhibitors (PSSRI), which includes the antidepressant paroxetine (**104**), showed efflux-related potential against *S. aureus*, particularly that conferred by NorA. In fact, paroxetine and its isomer, **105**, showed potency with respect to inhibition of this efflux pump against ethidium bromide. The isomer **105** also interfered with the accumulation of norfloxacin by NorA, reducing by 4- to 8-fold its MIC. [120] Femoxetine (**106**) is a paroxetine derivative, with activity as an EPI. [120] Studies were performed as to determine which moieties in paroxetine- and femoxetine-like PSSRI were detrimental for efflux pump inhibition in *S. aureus*, and

conclusions from SAR are highlighted in **Figure 11**. Synthesis of derivatives showed that the fluorine atom on the phenylpiperidine moiety is not associated with the activity. The *N*-substitution of the piperidine ring yields different kinds of activity, suggesting that the NH group forms a positive binding contact with the target pump, which does not happen if there is a *N*-substitution (compound **106**). The phenyl ether substituents also play an important role as determining the EPI activity. [121]

Also for this kind of compounds, a study was performed, in order to conclude whether the 4-phenyl ring is required for EPI activity and the effects of changes in the two-atom aryloxymethyl linker, replacing it with a thioether, amine, and alkene linker portions. Compounds **107** and **108** displayed the lowest MIC against *S. aureus*. Synthesis of multiple derivatives proved that the 4-phenyl moiety is not vital for EPI activity and that a variety of two-atom linker groups for the 3-aryl piperidine moiety can maintain EPI activity [122].

The ciclooxigenase-2 (COX-2) inhibitor celecoxib (**109**) has also received attention for its MDR inhibitory activity. There had been a link between COX-2 and *mdr1* gene expression, which has consequences in cancer chemo resistance, and the use of celecoxib (**109**) reversed drug resistance. If this is true for mammalian efflux pumps, for bacteria there is no evidence of a COX-2-like gene, suggesting that celecoxib acts differently in inhibiting MDR in bacteria. A study was carried out in order to prove this hypothesis, which demonstrated that this drug helped in increasing sensitivity to antibiotics in *S. aureus* and *Mycobacterium smegmatis*. These effects probably occur due to the blockage of MDR transporters involved in the efflux of antibiotics, such as ampicillin, ciprofloxacin, kanamycin, and chloramphenicol. The observation of the accumulation of ethidium bromide intracellularly leads to the conclusion that this compound acts as a NorA inhibitor [123].

As celecoxib (**109**) demonstrated such promising activity, analogues were tested, which bore low or none anti-inflammatory activity and possessed the 1,4-dihydropyrazolo[4,3-*c*]-benzothiazine-5,5-dioxide nucleus. Ethidium bromide efflux inhibition was tested first *in silico*, and the 17 molecules that fit the chemical space requirements were then reduced to six, based on the activity prediction. These compounds were then screened for their *in vitro* NorA inhibitory activity, using an overexpressing strain of *S. aureus*. One derivative in particular, compound **110**, showed the best activity concerning ethidium bromide efflux (76.9%) which is better than celecoxib (**109**) (64.5%). When in combination with ciprofloxacin, **110** displayed results comparable to reserpine (**30**) and better than paroxetine (**104**), which makes it a lead compound for the development of inhibitors of this kind [124].

Phenothiazine and thioxanthene derivatives are currently used as neuroleptic and antiemetic agents, and have been described as modest, but broad, antibacterial agents. Although their antibacterial activity is not enough to be clinically relevant, they have shown synergy with standard antibiotic agents, and have also been associated with the

inhibition of eukaryotic MDR efflux pumps, such as P-gp. The mechanism through which these compounds exert their antimicrobial potentiation is not yet fully understood. However, it is thought that it might have to do with the inhibition of efflux pumps [125]. A study focusing on the activity of several compounds of these families against *S. aureus* was carried out, with the main aim of showing their potential in inhibiting NorA. The compounds tested, chlorpromazine, fluphenazine, thioridazine, prochlorperazine (**111**), *cis*(*Z*)-flupentixol, and *trans*(*E*)-flupentixol (**112**), displayed intrinsic antimicrobial activity and good results when ethidium bromide efflux was assayed against a NorA overexpressing strains of *S. aureus*. Additionally, phenothiazine **111** and thioxanthene **112** were also able to reduce the proton motor force of *S. aureus*, by reducing the transmembrane potential [125].

Since the phenothiazine moiety proved itself to work as a template for the synthesis of new MDR EPIs, it was chosen to be the scaffold for derivatives with improved activity. The logic for these studies lied in the elimination of the structural features that were responsible for neuroleptic activity. As such, drastic modifications were made, such as the elimination of one ring of the tricyclic benzothiazine backbone and of the chain linked to the N-10 atom, a tertiary amine whose protonation is crucial for the interaction with the dopaminergic receptor, and the addition of a substituted phenyl ring at the C-3 position, to guarantee better lipophilicity. Therefore, a prototype of new 3-phenyl-2*H*-1,4-benzothiazines was developed. Preliminary screening on the derivatives using a wild-type *S. aureus* strain, both with and without ciprofloxacin, showed variable intrinsic and synergistic activity. Two compounds, **113** and **114**, displayed the best activity, while displaying no and weak antibacterial activity, respectively. These compounds were able to inhibit NorA efflux of ciprofloxacin, being suggested that **114** was even able to completely inhibit this pump [126].

Thioridazine (**115**), a phenothiazine, was also tested for its antibiotic potentiation, either in racemate or in its enantiomeric forms. All the forms were able to reduce the MIC of oxacillin in *S. aureus* and erythromycin in *Streptococcus pyogenes*, being the L-enantiomer more effective in the last case [127-128].

4. RND

RND pumps are found mostly in Gram-negative bacteria, being the most responsible pumps for MDR in this kind of organisms. They present a wide variety of lipophilic and amphiphilic substrates, which include several classes of antibiotics, antiseptics, dyes, and detergents. RND transporters present a unique tripartite complex, constituted by a minimum of twelve transmembrane segments: the transmembrane pump, the outer membrane channel, and the periplasmic adaptor protein, which puts the other two components in contact. This structural organization makes the extrusion of substrates directly into the external medium possible [32, 38]. Even though it has always been thought that RND transporters were exclusive to Gram-negative

bacteria, it has recently been identified and characterized FarE in *S. aureus*, homolog to the AcrB protein in *E. coli* [129-130].

Within this family, the most studied system is the AcrAB-TolC, from *E. coli*. This efflux pump is composed by three essential parts: a resistance-nodulation-division transporter, AcrB; a membrane fusion protein, AcrA; and a multifunctional outer membrane channel, TolC [7, 131-133]. It transports a wide variety of toxic compounds from the intracellular space directly into the medium, evading the periplasm, and using a proton gradient as the energy source [132-133]. *Pseudomonas aeruginosa* also possesses an efflux system of the RND family, the MexAB-OprM efflux pump, which confers resistance to a wide variety of antibiotics. This was the first and best characterized efflux system in bacteria. It was found that AcrB (Figure 12, PDB 4ZLJ) is homolog to MexB (Figure 12, PDB 2V50), being their structure very similar [7, 134].

Figure 13 comprises all the RND pump inhibitors that will be discussed in the following section.

4.1. RND inhibitors

4.1.1. Natural Products and Derivatives

One study revealed that the essential oil of *Helichrysum italicum* can reduce the resistance to chloramphenicol in *E. aerogenes*, *P. aeruginosa*, and *A. baumannii*. These data suggested that one or more EPIs are present within the essential oil since it was shown that geraniol (116) produced significant restoration of sensitivity to chloramphenicol by 16-fold, and rendered a total sensitivity of the organism when combined with of phenyl-arginine β -naphthylamide (PA β N), which will be discussed further ahead [135].

A study published by Aparna *et al.* (2014) had the aim of identifying natural compounds from plants that present efficacy in potentiating effects of antibiotics in *P. aeruginosa* overexpressing MexAB-OprM and *E. coli* overexpressing AcrAB-TolC through *in silico* virtual screening and pharmacophore approaches [136]. The compounds with the best *in silico* results were then tested for their EPI activity. The two compounds that showed the best activity in potentiating the activity of carbenicillin and levofloxacin in *P. aeruginosa* and *E. coli* were lanatoside C (117) and daidzein (118). Lanatoside C (117) is cardiac glycoside that inhibits the Na⁺-K⁺-ATPase, which can also be the explanation for its EPI activity against MexB and AcrB. Daidzein (118) is an isoflavone that has previously showed slight EPI activity in *Mycobacterium smegmatis* and as a modulator of P-glycoprotein, in human cervical carcinoma KB-V1 cells [136].

An *in silico* study by Ohene-Agyei *et al.* (2014) identified five phytochemicals that could be docked similarly to PA β N in the binding pocket of AcrB. Of these five, three of them were able to decrease resistance to antibiotics by inhibiting the AcrAB-TolC system: plumbagin (119) increased sensitivity to erythromycin, chloramphenicol, and tetraphenylphosphonium; nordihydroguarectic acid (NDGA)

(120) potentiated the activity of erythromycin, chloramphenicol, tetraphenylphosphonium, novobiocin, and tetracycline; and shikonin (121) decreased resistance to tetraphenylphosphonium [137].

A study proved the usefulness of curcumin (64) as a RND EPI at a concentration of 50 μ g/ml, increasing the sensitivity of carbenicillin, ceftazidime, and meropenem in *P. aeruginosa*, in decreasing order. It also managed to circumvent the resistance to gentamicin and ciprofloxacin in resistant isolates, which PA β N failed, leading to the assumption that curcumin inhibits efflux pumps differently from PA β N [138].

A screening of 85 000 microbial fermentation extracts derived from 3 600 strains of actinomycetes and 3 500 strains of fungi was made, with the aim of discovering EPIs that increased sensitivity of levofloxacin in *P. aeruginosa* overexpressing the MexAB-OprM or the MexEF-OprN pump. Two compounds, EA-371 α (122) and EA-371- δ (123), produced by a strain of *Streptomyces* sp., demonstrated inhibitory activity against MexAB-OprM [139].

4.1.2. Synthetic Compounds

The first compound with potent inhibition of RND efflux pumps was PA β N (124), a dipeptide amine. This dipeptide was identified as a hit compound from the screening of 200 000 samples of small molecules that potentiate the activity of antibacterial levofloxacin against strains of *P. aeruginosa* that overexpressed MexAB, MexCD and MexEF pumps [28, 140]. Compound 124 is a peptidomimetic, developed for using in adjunctive therapy. This compound is a substrate for RND pumps, acting as a competitive inhibitor of multidrug efflux systems, such as MexB, MexD, and MexF, binding to the substrate pocket, impeding antibiotic binding and/or extrusion. Particularly, this EPI binds in a location close to the antibiotic binding site, generating steric hindrance, making it more difficult for the antibiotic to bind. In other words, less quantity of antibiotic is exported, making its intracellular levels higher, thus restoring antibiotic sensitivity [11, 25, 27-28, 141-143]. However, 124 can affect the integrity of the membrane when used in high concentrations, making it prone to the emergency of resistant profiles, namely the modification of the lipopolysaccharide structure, leading to changes in drug penetration [24].

PA β N (124) has been validated against the AcrAB-TolC in *Klebsiella pneumonia*, *E. coli*, *Salmonella enterica* serovar Typhimurium and *Enterobacter aerogenes*, and in multiple homologous systems including *Acinetobacter baumannii*, *Campylobacter jejuni*, and *Campylobacter coli* [11, 142]. Its potency has been demonstrated against the norfloxacin resistance conferred by the Mex efflux system of *Pseudomonas aeruginosa*, the AcrAB efflux systems of the Enterobacteriaceae, and the erythromycin efflux system of *C. jejuni* [19].

Studies of SAR demonstrated that the basic middle amino acid, arginine, was detrimental for activity, although a substitution with L-lysine showed the same potency as the

lead, and the introduction of L-ornithine, originating compound L-Phe-L-Orn- β -Na, showed a 2-fold increased potency. As the ornithine moiety provided a simpler synthesis, it became the standard middle residue in successive compounds [25, 142, 144]. However, these compounds were not stable upon incubation with human serum, due to the occurrence of cleavage of peptide linkage between both amino acids, which is explained by the fact that natural (L) amino acids are contained within its structure. In order to overcome this problem, methylation of the NH that links both amino acids was achieved, maintaining potency and being this derivative able to resist serum proteases. Serum stability was also increased by replacing the L-amino acid with D-amino acid [25, 142]. In the same study, the authors also proved that the amino acid in the *aal* position (**Figure 13, 124**) should be appropriately substituted, and the replacement of phenylalanine with a homophenylalanine led to improved potency. The problem with this compound was the fact that the ornithine moiety could easily form a lactam, rendering the compound inactive. This was overcome by switching the L-ornithine with the L-phenylalanine, which originated L-Orn-L-hPhe- β -Na, which originated a compound just as potent, but with no propensity to form a lactam. As for the β -aminonaphthalene moiety, it was proven that replacing it with 3-aminoquinoline led to reduced cytotoxicity and intrinsic antibacterial activity, even though its potency was slightly reduced [25, 142]. These efforts originated the compound L-Orn-L-hPhe-3-NHQ (**125**). In conclusion, the analogues synthesized by Renau *et al.* (1999) did not show improvements in potency when compared to PA β N (**124**), but showed increased stability [142].

The same group developed further studies with this type of molecules and demonstrated that conformational restriction of the amino group of ornithine showed less toxicity, while maintaining potency. Such compounds were attained by adding a proline substituent, thus incorporating the two basic residues necessary for activity, which resulted in the successful synthesis of constrained derivatives, more potent and less toxic than **125**. The most interesting derivative, compound **126**, was as potent as **125**, but more than 4-fold less toxic. This compound also retained the activity against RND efflux pumps MexAB-OprM, MexCD-OprJ and MexEF-OprN. Although this compound showed similar protein binding in rats as **125**, its pharmacokinetic parameters were improved, and its efficacy in combination with levofloxacin was demonstrated in an *in vivo* model [145].

In a latter study developed by the same group, several non-peptide analogues were synthesized, and it was hypothesized that the peptide backbone is not essential for the activity of this type of compounds. The results suggested that the inhibition of pumps was possible due to the di-cationic nature of the compound, the appropriate lipophilicity and a disposition similar to that of compound **125** [146].

MBX2319 (**127**) is a pyranopyrimidine EPI inhibitor of AcrAB-TolC efflux pump in *E.coli* and other Enterobacteriaceae [141]. Compound **127** did not show any

antibacterial activity, which is a positive feature for EPIs. It also potentiated the antibacterial activity of fluoroquinolones, such as ciprofloxacin and levofloxacin, and β -lactams, such as piperacillin against strains of *E. coli* that presented AcrAB-TolC efflux pumps [28, 141]. In *P. aeruginosa*, although compound **127** presents activity against RND-type pumps, it is unable to penetrate the outer membrane, which is highly selective [28].

In *E. coli*, the target of compound **127** is the membrane transporter AcrB. This molecule binds to the hydrophobic trap of the T protomer, interacting with the hydrophobic residues that constitute the deep binding pocket as a hydrophobic trap. π - π Interactions also occurs, between the pyridine ring of **127** and the aromatic side chain of Phe-628. The phenyl and morpholinyl groups also interact with Phe-178 and Phe-615. The dimethylenesulfide linker and the geminal dimethyl group present van der Waals interactions between the side chain of Phe-178, and Tyr-327 and Met-573, respectively [28, 147].

SAR studies demonstrated that the maintenance of activity depended on the geminal dimethyl moiety of the tetrahydropyran, the nitrile group and the length of the dimethylenesulfide linker. This data is presented in **Figure 14**. On the other hand, the morpholine moiety and aryl group are both prone to substitution, originating more stable derivatives in terms of activity, solubility, and metabolism [28]. Studies with this scaffold have shown that non-acidic substituents can be added to the phenyl group, in order to improve potency and CYP450 inhibition, and modifications to the morpholinyl group has greater effects on solubility and stability [140].

Analogues were synthesized by Sjuts *et al.* (2016), by changing the structure and functional groups of compound **127** (148). They reached the conclusions that the introduction of a 2,6-dimethyl to the morpholinyl group improved both microsomal stability and EPI activity. The replacement of the morpholinyl group with 2-methoxyethylpiperazinyl improved aqueous solubility, at the cost of a slight reduction of EPI activity. Two analogues, **128** and **129**, resultant of the combination of 2,6-dimethylmorpholinyl and acetamide or acrylamide, respectively, on the phenyl group, resulted in a 10-fold to 20-fold increase in EPI activity against *E. coli*, respectively, comparing to MBX2319 (**127**) [28, 148]. The increased EPI activity of these compounds has its basis in the acetamide and acrylamide groups and the morpholinyl group. The acetamide groups are engaged in highly ordered and complex hydrogen bonds, with its center in a solvent water molecule, which plays the role of a hydrogen bond donor to the carbonyl backbone oxygen of Ala-286 and Gln-151 side chain of AcrB. The acrylamide group forms a bridging hydrogen bond through a water molecule to the side chain of Gln-176 [28].

Nguyen *et al.* (2015) also synthesized a series of analogues, with the morpholine scaffold substituted by a 2,6-dimethylmorpholinyl group (140). Many of these analogues exhibited a solubility improvement of 10-fold, compared to compound **127**. The most potent and promising compounds

of these series were compounds **130**, **131**, **132**, **133** and **134** [140].

Through a screening of an *N*-heterocyclic organic compound library, Bohnert and Kern (2005) were able to find a novel class of EPIs, the arylpiperazines [25, 149]. The aim of their work was to find compounds that would potentiate levofloxacin potency against strains of *E. coli* overexpressing *acrAB* and *acrEF*. This was fulfilled when they found phenylpiperazine derivatives with promising activity [25, 149]. SAR studies suggested that elongation of the spacer between the benzene ring and the piperazine ring would enhance potency. Also, substitutions on the benzene ring by halogen atoms led to an increase in potency [24, 149]. On the other hand, the potency would decrease when an ethyl or phenyl group was added to the piperazine ring. With this, the authors found the most potent phenylpiperazine, *meta*-TFMPP (**135**), and naphthylpiperazine, 1-(1-naphthylmethyl)-piperazine (NMP) (**136**) [149].

NMP (**136**) was the most potent unsubstituted arylpiperazine, able to increase the intracellular concentration of chloramphenicol, tetracycline, linezolid, fluoroquinolones, and macrolides. Although it does not display action in *P. aeruginosa*, it shows effectiveness in *A. baumannii* and several Enterobacteriaceae [11, 23]. EPI activity was also reported in clinical isolates of *E. coli*, most relevantly in fluoroquinolone resistance [150]. Through computational analysis, it was found that NMP (**136**) binds with high affinity to the lower part of the deep pocket of the B monomer, overlapping in part the binding site of chloramphenicol [11]. The binding site for NMP (**136**) includes interactions with hydrophobic residues near the hydrophobic patch and Gly-617 of the G-loop. This data suggests that NMP (**136**) interferes with the movement of the G-loop, important for extruding substrates, thus inhibiting the action of AcrB [25].

Nakayama *et al.* (2003) performed a series of syntheses in order to attain a compound specific for the inhibition of a MexAB-OprM specific EPI [151-152]. They identified a hit, compound **137**, through high-throughput screening for levofloxacin potentiation using a strain of *P. aeruginosa* strain overexpressing MexAB-OprM. The features this group was looking for was the effective inhibition of efflux pump and the lack of intrinsic antibacterial activity, since efflux pumps are not vital for the organism's survival [151-152]. It was shown that this compound effectively potentiated the activity of levofloxacin, leading to an 8-fold decrease in the antibiotic's MIC. However, its physicochemical properties were problematic: water solubility was poor and it presented high affinity to serum albumin. Therefore, they divided the molecule into three parts, A, B, and C (**137**) and looked for alternative scaffolds and substituted polar groups, in order to reduce serum protein binding [152].

It was shown that when a carboxamide was introduced in A, the affinity to serum albumin was diminished, as was its activity. More polar moieties led to a complete loss of activity. The strategy for the styrene portion (B) that was the most successful was to substitute the benzene ring to a 3,5-

disubstituted pyridine, which had both good activity and no major serum influence. As for the vinyl group in B, it was demonstrated that molecules bearing methoxy and ethylene moieties displayed favorable activity profiles. As for part C, the basis of the modifications was the fact that the introduction of bulky or hydrophilic groups adjacent to the carboxyl group would reduce protein binding. An enantiomeric pair containing a dioxolane moiety presented both activity and serum stability, being the *S* enantiomer more active [152].

Regarding the general scaffold, new compounds were designed, keeping in mind that the thiazole moiety was crucial for activity. Therefore, a molecule having a thiazole side chain attached to the C-7 position of a quinolone was synthesized. Substitution with a tetrazole portion yielded better results than substitution with a carboxylic acid. One compound (**138**) presented the best activity and serum stability [153].

Further efforts were placed in order to increase the potency of this compound. The *in vitro* activity was enhanced by the introduction of a hydrophobic group at the 2-position of the pyridopyrimidine scaffold, and hydrophilic substitution would not compromise the activity. The most promising compound had the inclusion of an olefin spacer between the tetrazole and the pyridopyrimidine scaffold, with the limitation of being prone to photoisomerization [154]. To circumvent this, the ethylene tether between the pyridopyrimidine was replaced with an amide bond, providing stability against photoisomerization and, unexpectedly, improving potency. However, another problem arose: the lack of solubility for intravenous use [155]. The following step was the introduction of a secondary amine, giving a zwitterionic analogue, with retention of activity. C-2 Analogues, namely with cyclic substituents, also showed promising results [156]. Once again, the problem of solubility arose. Therefore, efforts were placed in making the molecule more hydrophilic, with the introduction of substituted aromatic moieties. Analogues 4-substituted were the most potent, and the introduction of a morpholine moiety led to increased solubility, maintaining the serum stability. Compound **139** was the most balanced molecule in terms of the desired features [157]. The last refinement made was the introduction of a quaternary ammonium salt, which led to D13-9001 (**140**). Adding to its high solubility and safety, it was able to potentiate the activity of aztreonam [158]. **Figure 15** illustrates the SAR established for **140** and derivatives.

3D Crystal structures of **140** bound to the target, either AcrB or MexB, show that the *tert*-butyl thiazolyl aminocarboxyl pyridopyrimidine moiety of this compound binds tightly to a narrow depression, designated by hydrophobic trap, close to the deep substrate binding pocket. Additionally, the tetrazole ring and the piperidine acetoamino ethylene ammonio-acetate moiety interact with ionic and/or hydrophilic residues in the substrate translocation channel. The piperidine acetoamino ethylene ammonio-acetate moiety also covers part of the minocycline and doxorubicin binding site [23, 28, 151].

Quinoline derivatives with EPI activity include a multiplicity of derivatives, including pyrido-, alkoxy-, thioalkoxy-, alkylamino- and chloro- derivatives. These compounds were able to restore an intracellular concentration of antibiotic drugs expelled by efflux pumps, as well as to induce the increase in antibiotic susceptibility, acting as competitive inhibitors of the antibiotic flux. In *E. aerogenes* and *K. pneumoniae*, quinolines were more effective than PA β N (**124**) in inhibiting the activity of the AcrB pump, potentiating the activity of fluoroquinolones, tetracycline, and chloramphenicol [11, 24, 26]. Among the different classes of substituted quinolines, it was proven that the best side chain was piperidinoethyl, which potentiated efficiently the restoration of drug susceptibility in alkoxy- and thioalkoxyquinolines. The heteroatom connecting the side chain is also crucial for activity, being the most potent the amino substituent, then the thioalkyl-, followed by the oxo-derivative. The position of the branched substituted groups is also detrimental for the EPI activity [24]. Branched side chains containing piperidinoethyl and morpholinopropyl groups were associated with the most interesting results, being compound **141**, containing a piperidinoethyl side chain, the most active when in combination with chloramphenicol [159]. It has been hypothesized that the pump inhibition is greatly influenced by the branched side chain, and takes place either in the inner-membrane transporter or at the junction between the inner pump and the outer channel [159].

Given these promising results, further research in this kind of compounds was performed. Alkoxyquinolines were synthesized and tested against a strain of *E. aerogenes* overexpressing the AcrAB efflux pump. Compound **142** induced an increase in the susceptibility of chloramphenicol, tetracycline, and fluoroquinolone. This could happen due to interference during active pumping out of the antibiotic [160].

Chloroquinolines also demonstrated efficacy as EPIs, modulating chloramphenicol activity, decreasing its MIC 8-fold for resistant strains. Synthesis of analogues led to the conclusion that amino derivatives are more potent than thiol derivatives, pointing out the importance of the side chain for the efflux inhibition. Protonation of the nitrogen could also play an important role at environmental pH, stimulating recognition and binding of the drug to the sites located within the pump's cavity. The heteroatom is also crucial for the activity, being nitrogen the most efficient. Compound **143** is the chloroquinoline with the best results found for the inhibition of the main efflux pump of *E. aerogenes* [161].

Alkylaminoquinazoline derivatives lacking a nitro group were also evaluated for their ability to decrease chloramphenicol and quinolone efflux in *E. aerogenes*, through the inhibition of AcrAB efflux pump. The results obtained suggest that this family of compounds recognizes the same pump site responsible for the transport of chloramphenicol and quinolones, competing with them. Structurally, it has been shown that a morpholine group with a propyl chain makes a more active alkylaminoquinazoline.

The most active compound of this family is compound **144** [162].

Benzothiazoles 2-substituted were first tested for the potentiation of ciprofloxacin in AdeABC overexpressing *A. baumannii*. These compounds did not show antibacterial activity when tested alone in bacteria. However, when combined with ciprofloxacin, there was an observation of a reversal in antibacterial susceptibility. The synthesis of several derivatives of this family led to pharmacophore studies, regarding the features of the most promising compounds. Using computational methods, it was revealed that, in order for the compounds to be biologically active, the following features should be present: the nitrogen atom in the thiazole ring and the carbonyl oxygen in the amide function substituted on the second position of the benzothiazole ring must have an hydrogen bonding acceptor property; the benzene ring in the fused ring system, the phenyl group attached to the second position of the benzothiazole ring and the phenyl ring in the 2-phenylacetamide and/or 3-phenylpropionamide moiety are essential, since they provide a hydrophobic aromatic property. Of the synthesized compounds, the ones that gathered these features and, therefore, presented the most promising activity, were compounds **145**, **146**, and **147** [163]. These compounds were also tested for their activity against AcrAB-TolC, in an overexpressing strain of *E. coli*, reducing the MIC of ciprofloxacin 10-fold, in the case of **146** and **147**, and 8-fold, for **145** [164]. Unlike AdeABC, AcrAB-TolC's crystal structure is elucidated, making docking studies possible. This predicted that all three compounds act as AcrB substrates, binding to the distal pocket site in the AcrB porter domain by blocking or inhibiting the ciprofloxacin binding site, generating steric hindrance and thus impeding the binding of the antibiotic. Furthermore, **146** and **147** showed stronger binding interactions than ciprofloxacin [164].

Indole derivatives have been useful in inhibiting protein targets of virus, tumors and bacteria. Since small molecules with conjugated aromatic rings, in which indoles fit, present high potential as EPIs, efforts were placed in synthesizing derivatives that presented this kind of activity. The TolC 3D structure has already been disclosed, making possible the structure-based design of inhibitors. Indoles **148** and **149** were proven efficient inhibitors of TolC in *E. coli*, potentiating the effects of chloramphenicol, tetracycline, erythromycin, and ciprofloxacin, with decreased MIC values of 2-fold for tetracycline and erythromycin and 8-fold for the other two [165].

4.1.3. Existing Drugs

Other drugs used for treatments other than antibacterial have demonstrated EPI activity. Tetracycline resistance in *P. aeruginosa* was reduced 8-fold when the phenothiazine fluphenazine, an antipsychotic, was present. Chlorpromazine (**150**) also showed EPI-like activity for the BpeAB-OprB system in *Burkholderia pseudomallei* and a reduction of the expression of *acrB* in *E. coli* and *S. enterica* samovar

1 Typhimurium, suggesting an inhibition of AcrB production
2 [11, 27, 128].

3 Selective serotonin-reuptake inhibitors have been studied for
4 their ability to act as EPIs for RND efflux pumps in *E. coli*.
5 It was found that sertraline (**151**) was capable of potentiating
6 the activity of fluoroquinolones. However, its activity is
7 limited, since it is possible that this compound also acts as an
8 inductor of efflux pumps. Studies have shown that this
9 compound could accumulate in tissues or cells, and
10 therefore, sertraline's (**151**) concentration may be higher
11 than that measured in plasma. This means that a standard
12 dose of this drug could enhance antibiotic efficacy. It has not
13 yet been studied if a sertraline (**151**) metabolite could be
14 administered at a dose higher than sertraline. However, it
15 was hypothesized that it could have a better EPI activity,
16 while causing less undesired effects on the central nervous
17 system [166].

18 Through the observation of previously reported EPIs,
19 Piddock *et al.* (2010) suggested that simple heterocyclic
20 nitrogen-containing compounds could also act as EPIs and
21 synergize with ciprofloxacin in strains of *S. enterica* serovar
22 Typhimurium that overexpressed the AcrAB-TolC efflux
23 pump [167]. It has been shown that compounds containing a
24 phenylethylamine moiety, as PA β N (**126**) does, such as
25 epinephrine, norepinephrine, and cathinone, are able to
26 potentiate the activity of ciprofloxacin. Of the compounds
27 tested, trimethoprim (**152**) and epinephrine (**153**), showed
28 the best results for the tested strains, which included, apart
29 from *S. enterica* serovar Typhimurium, *P. aeruginosa*, *E.*
30 *coli*, and *K. pneumoniae*. These compounds showed synergy
31 for the strains where AcrAB-TolC was active, but showed no
32 activity when the pump was inactive. Thus, it can be
33 hypothesized that these compounds either inhibit the
34 expression of the genes encoding AcrAB-TolC, or interact
35 directly with the pump [167]. Both compounds possess
36 structural features similar to previously described EPIs, such
37 as PA β N (**124**), L-Orn-L-hPhe-3-NHQ (**125**), and NMP
38 (**136**), such as an aromatic ring linked to a basic nitrogen,
39 in the case of epinephrine (**153**), or a pyrimidine ring, for
40 trimethoprim (**152**). Trimethoprim (**152**) is already used in
41 combination with another antibiotic, sulfamethoxazole, and
42 combination with a quinolone was hypothesized to be a
43 viable choice. On the other hand, a combination of
44 epinephrine (**153**) with an antibiotic does not seem a viable
45 option, because of the likelihood of the occurrence of
46 sympathomimetic effects [167].

47 The antimalarial artesunate (**154**) has also shown EPI
48 activity, enhancing the effects of β -lactam antibiotics, such
49 as penicillin G, oxacillin, and ampicillin, and novobiocin
50 against *E. coli*. The mechanism through which **154** expresses
51 its activity is by the inhibition of the expression of the
52 AcrAB-TolC system, not exhibiting antibiotic activity by
53 itself [168].

56 5. SMR

57 The SMR family comprises the smallest drug efflux proteins
58 known, possessing only four transmembrane segments in its

composition, and are exclusive to bacteria. They are
involved in the efflux of lipophilic compounds, such as
quaternary ammonium salts and a variety of antibiotics.
These pumps also use an electrochemical proton gradient in
order to exert its efflux activity [7, 32, 38, 169].

One example of a SMR-type pump is the EmrE transporter
(**Figure 16**, PDB 3B61), present in *E. coli*. In the absence of
the ligand, the asymmetric unit of EmrE contains eight EmrE
monomers, with four transmembrane helices composing
each of them. If a ligand is present, it can function as an
inverted homodimer [7, 170].

There have not been many studies concerning inhibitors of
this family of efflux pumps, and the few that have been
performed were only *in silico*. **Figure 17** shows the
structures of the SMR inhibitors that will be further
discussed.

5.1. SMR inhibitors

5.1.1. Natural Products

Quercetin (**155**) is a flavonol, present in many plants. This
compound is very well tolerated in humans, as are its
glycoconjugates, and presents a multiplicity of activities,
such as antioxidant, anti-inflammatory and
antimycobacterial. Only docking studies of this compound
with two pumps belonging to the SMR family, the Mmr, in
Mycobacterium smegmatis, and the EmrE pumps, in *E. coli*,
were carried out. In Mmr, the hydroxyl groups present in the
3' and 4' positions of the B ring of quercetin were predicted
to interact with Glu-14 in the helix 1, and the keto group of
the hydroxychromen ring of the ligand interacts with the
Trp-48 of the helix 1. Additionally, hydrophobic interactions
were shown by the residues Phe-44, Tyr-40 and Trp-48, and
 π - π interactions were found with Phe-44. Furthermore, **155**
was also predicted of interacting with dimeric forms of the
protein [171]. In the case of EmrE, the interaction between
the 3' and 4' hydroxyls with Glu-14 can also be observed, as
can the hydroxyl in the C-7 position with Tyr-60. Hydrophobic
interactions are present, with the residues Ile-
11, Ser-64, Gly-67 and Leu-70, and π - π interactions exist
with the residues Tyr-60 and Trp-63. Structural studies
suggest that EmrE is mostly in the dimer state, which also
interacts with quercetin (**155**) [171].

5.1.2. Synthetic Compounds

Cation ligands were subjected to docking studies with the
Mmr protein of the SMR family in *Mycobacterium*
tuberculosis and the EmrE dimer from *E. coli*. The rationale
behind this study is the fact that SMR can extrude cations.
This study led to the identification of new virtual leads,
amongst which is compound **156**. ADME studies prove that
all the molecules present in this study obey the Lipinski rule
of five and the Jorgenson rule of three, which shows that the
compounds possess good drug-like properties [172].

6. MATE

MATE transporters are responsible for the efflux of cationic, lipophilic substances, providing bacteria and cancer cells with MDR, using Na⁺ or H⁺ influx. They have 12 transmembrane helices, constituted by 400 to 700 amino acids. The mechanism of efflux is thought to happen by a rocker-switch mechanism. Members of the MATE family include the MepA (**Figure 18**, PDB 1TZP) transporter, in *S. aureus*, even though seventeen MATE proteins have been identified in eleven species, capable of extruding antibiotics. Ethidium bromide, tetraphenylphosphonium, acriflavine, berberine, and norfloxacin have been reported as MATE substrates [38, 173-181].

In **Figure 19** are compiled the structures of the compounds that have so far proven their usefulness as MATE inhibitors.

6.1. MATE inhibitors

6.1.1. Natural Products

A study performed by Keshewani *et al.* (2017) accomplished the docking of phytochemicals with the NorM transporter, belonging to the MATE family [182]. After a screening of a library of natural products, three hits were identified: derhamnosyl suspenaside (**157**), prunin 7''-O-gallate (**158**), and quercetin diglucoside (**159**). These were the best compounds, as their results in binding free energy calculation were the most favorable. When binding free energy decomposition and hydrogen and hydrophobic statistics were taken into account, **158** was elected the best hit compound. Furthermore, this compound was stable during the simulation. Overall, it has been suggested that the compounds which interact towards the cation binding residue in the central cavity of NorM will have better results, and that good inhibitors should have interaction with a few key residues, Gln-34, Val-35, Gly-38, Phe-63, Tyr-67, Ile-68, Glu-261, Tyr-294, and Asp-377, which will lead to maximum stability and affinity with the NorM transporter [182].

6.1.2. Synthetic Compounds

These compounds have been previously described as MFS inhibitors, namely of the NorA pump. However, similar activity has been described for the MepA pump, of the MATE family. Compound **80** (**Figure 5**), incorporating a bisaryl urea moiety at the 7-position, was able to successfully inhibit approximately 84% of ethidium bromide efflux in a MepA overexpressing strain of *S. aureus* [104].

Phenylquinoline derivatives also proved efficient against the same strain. Differently from what was observed with NorA, the compounds that inhibited MepA most efficiently were **86** (**Figure 5**) and **160** (**Figure 19**), which were able to reduce ethidium bromide's MIC by 16-fold at concentrations of 0.78 and 1.56 µg/ml, respectively, in the MepA overexpressing strain. Ethidium bromide was chosen, instead of ciprofloxacin, as it is a better MepA substrate and other derivatives were able to inhibit both NorA and MepA efflux pumps, being able to completely restore the activity of

ciprofloxacin and ethidium bromide against strains overexpressing such pumps [107].

Paroxetine derivatives (**104**), effective against the NorA pump, also demonstrated potency in inhibiting the MepA pump in *S. aureus*. Oppositely to NorA, paroxetine derivatives do not need the fluorophenyl ring at position 4 to be MepA EPIs. In fact, 4-unsubstituted analogues proved to be more potent than 4-F-phenyl derivatives in inhibiting the MepA pump, as is the case of compound **161** and **162** [122].

Efflux pumps are one of the main causes for antimicrobial resistance. Their inhibition can lead to the restoration of bacteria susceptibility and antibiotic efficacy. Throughout this review, the data presented leads to the conclusion that EPIs are promising compounds and can help reversing the problem of antimicrobial resistance. However, the research into these kind of compounds is still in a very preliminary state, since no EPI is yet under clinical trials.

Even though no general structural features can be pointed out for a molecule to be an EPI, it can be observed that small molecules with high lipophilicity are most likely to fulfill the requirements to inhibit efflux pumps. Hydrophilic compounds can also be transported through porins present in the membrane. Heterocyclic compounds also show potential as EPIs, mainly nitrogen heterocycles, but also fluorine and boron heteroatoms, since the likelihood of bacterial exposure to these atoms is very low. The basis for the MDR pumps inhibition has to do with the penetration rules, and whether or not a compound is capable of penetrating the bacterial membrane. Therefore, a library of compounds that are able to do that would be something that would bring advances to this study field.

One of the main aims of this review is to present SAR studies, so that pharmaceutical chemists can refine molecular structures, leading to the achievement of more potent and specific compounds. The use of synthetic approaches, either to modify molecules obtained from natural sources, or drugs that are already in therapeutic use, or even to develop novel compounds, is vital for these SAR studies. The search of EPIs derived from natural products comes from the fact that plants need to protect themselves from pathogenic microorganisms, and produce metabolites that act as antimicrobial and EPIs, that synergize with each other. However, the referred compounds are not specific for the pump they have been studied, showing a multitarget character: flavonoids, coumarins, cinnamic acids, among others, present a multiplicity of activities already described, suggesting their promiscuity.

The advantage of assays with existing drugs lies in their safety profile and, most times, their clinical use for combination therapy has been characterized. In some cases, the compounds were even investigated as inhibitors of human efflux pumps, for cancer treatment. This can be regarded as an advantage, leading to a new class of drugs with dual human/bacterial EPI, particularly useful in cancer patients with infections, potentiating the effects of both the anticancer and the antibacterial drugs.

The future in studies in bacterial EPIs should lie either in the search for selectivity for the bacterial target, or in the disclosure of the common features of the universal bacterial EPI, that can successfully target all efflux pumps. The SAR studies gathered herein with the 3D structural information available are expected to accelerate the structure-based design of new EPIs, founded on the existing models.

LIST OF ABBREVIATIONS

3D-QSAR: Three-dimensional quantitative structure activity relationship; ABC: ATP-binding cassette; ADME: Absorption, distribution, metabolism and excretion; ATP: Adenosine triphosphate; BCRP: Breast cancer resistance protein; COX-2: Cyclooxygenase-2; CYP450: Cytochrome P450; EPI: Efflux pump inhibitor; IC₅₀: Half maximal inhibitory concentration; MATE: Multidrug and toxic compound extrusion; MDR: Multidrug resistance; MIC: Minimum inhibitory concentration; MFS: Major facilitator superfamily; MRP: Multidrug resistance protein; MRSA: Methicillin-resistant *Staphylococcus aureus*; NDGA: Nordihydroguaric acid; NMP: 1-(1-Naphthylmethyl)-piperazine; P-gp: P-Glycoprotein; PAβN: Phenyl-arginine β-naphthylamide; PDB: Protein Data Bank; PSSRI: Phenylpiperidine selective serotonin reuptake inhibitors; RND: Resistance-nodulation-division; SAR: Structure activity relationship; SMR: Small multidrug resistance.

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Title page

Medicinal Chemistry Updates on Bacterial Efflux Pump Modulators

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Medicinal Chemistry Updates on Bacterial Efflux Pump Modulators

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Abstract: Antibiotic resistance is one of the most pressing health issues of our days. It can arise due to a multiplicity of factors, such as target modification, decrease in the drug uptake, changes in the metabolic pathways and activation of efflux pumps. The overexpression of efflux pumps is responsible for the extrusion of drugs, making antibiotic therapy fail, as the quantity of intracellular antibiotic is not enough to provide the desired therapeutic effect.

Efflux pumps can be included in five families according to their composition, nature of substrates, energy source, and number of transmembrane spanning regions. The ABC superfamily are mainly found in Gram-positive bacteria, use ATP as an energy source, and only a limited number of ABC pumps confer multidrug resistance (MDR).

On the other hand, the MFS family, most present in Gram-positive bacteria, and the RND family, characteristic of Gram-negative bacteria, are the most associated with antibiotic resistance. A wide variety of inhibitors have been disclosed for both families, from either natural or synthetic sources, or even drugs that are currently in therapy for other diseases.

The other two families are the SMR, which are the smallest drug efflux proteins known, and the MATE family, whose pumps can also resort to the sodium gradient as an energy source.

In this review, it is intended to present a comprehensive review of the classes of efflux pump inhibitors from the various sources, highlighting their structure-activity relationships, which can be useful for medicinal chemists in the pursuit of novel efflux pump inhibitors.

Keywords: Antimicrobial resistance; efflux pump inhibitors; natural products; synthetic compounds; existing drugs; Structure-activity relationship.

1. INTRODUCTION

Bacteria were discovered the late 19th century as infectious agents. It was, however, half a century later that antibiotics completely revolutionized medicine and became a turning point in human history. Unfortunately, the misruled use of these drugs led to the appearance of resistant strains, and to the concern of a return to the pre-antibiotic era, where the simplest, most treatable infections can become deadly [1-2]. Antibiotic resistance can arise due to multiple causes. The most common are the overuse and/or inappropriate prescribing, as well as extensive veterinary and agricultural use. These factors are especially alarming if it is taken into account the very few new antibiotics that were discovered in the past years [3]. Due to their genetic plasticity, bacteria can develop defense mechanisms in response to environmental threats. As far as antibiotics are concerned, bacteria use two major strategies to adapt to these drugs, which are mutations

in genes and the acquisition of resistance genes through horizontal gene transfer. The first strategy can be manifested through modification of the target, decrease in the drug uptake, activation of efflux pumps to extrude the drug or changes in metabolic pathways. The latter is related to the acquisition of external genetic material through transformation, transduction, or conjugation [4-5]. Recently, vesicle-mediated resistance has also been regarded as a mechanism of resistance acquisition [6].

Efflux pumps are ubiquitous in bacteria, and can be encoded by genes present either in the bacterial chromosomes or in plasmids [7-8]. They concern five families, according to their composition, substrates, energy sources, and number of transmembrane spanning regions: the adenosine triphosphate (ATP)-binding *cassette* (ABC) superfamily, the resistance-nodulation-division family (RND), the major facilitator superfamily (MFS), the small multidrug resistance (SMR)

family, and the multidrug and toxic compound extrusion (MATE) family (**Figure 1**) [7-12].

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Efflux pumps can be further divided into two major families, the primary transporters, which hydrolyze ATP as a source of energy, as is the case of the ABC family, and secondary transporters, which use the proton or sodium gradient as a source of energy [13]. Overexpression of multidrug efflux pumps is correlated with an increase in the minimum inhibitory concentration (MIC) of antibiotics, and thus, these pumps can be considered potential antibacterial targets.

Therefore, the development of efflux pump inhibitors may be a way of fighting against resistant microorganisms. Studies have been carried out on the structure of efflux pumps, as well as on their mechanisms of binding and extrusion, leading to the possibility of structure-based design of efflux pump inhibitors (EPIs). The rationale for the development of these EPIs could be, for instance, the inhibition of the energy sources required for the ability to extrude antibiotics, and the development of compounds that compete with the antibiotics for their extrusion. However, the main challenge lies in the fact that many compounds that present EPI activity and are already used for the treatment of other diseases are toxic for human cells in the concentrations required to present EPI activity and, therefore, clinically useless [7-9, 13].

Even though there are already several reviews which describe different classes of EPIs [11, 13-31], most focus on a particular source of compounds or on a class of pumps or bacteria. Herein, it is intended to present a comprehensive review of the several classes of EPIs from natural and synthetic sources and from existing drugs in therapeutics and to highlight structure-activity relationship studies in the referred efflux pumps (ABC, RND, MFS, SMR and MATE), which can be useful for medicinal chemists in the pursuit of novel compounds for this goal.

2. ABC

The ABC transporters are primary transporters, and they exist in every membrane systems, whether the organism is prokaryotic or eukaryotic. P-Glycoprotein (P-gp) is a very well known human ABC transporter, involved in the resistance of cytotoxic drugs used in cancer therapy [32-34], whose modulation has been regarded as a study field [35-37]. Structurally, an ABC transporter consists of four conserved domains, two transmembrane domains, and two cytoplasmic domains, which bind to nucleotides and, therefore, are involved in ATP binding [32, 38]. In fact, the nucleotide binding domains are responsible for hydrolyzing ATP, which will lead to conformational changes in the attached transmembrane domains, making it possible for

substrates to cross the lipid bilayer of the membrane, either in an inwards or an outwards fashion [39].

The bacterial transporters are highly selective for sugars, amino acids, metallic cations, organo-iron complexes, vitamins, and antibiotics [32, 40]. They are found in Gram-positive bacteria, such as *Enterococcus faecalis* and *Staphylococcus aureus*, and confer resistance to macrolides and bacitracin. However, only a limited number of ABC pumps are associated with MDR, which means that ABC pumps do not play a pivotal role in conferring antibiotic resistance in bacteria [32-33].

As such, bacterial ABC transporter inhibitors are scarce. The few EPIs discovered in this context are mainly against MsrA (Figure 2, PDB 1NWA), whose tridimensional structure has been elucidated [41].

The structures of the ABC inhibitors that will be further detailed throughout this section are comprised in Figure 3.

2.1. ABC inhibitors

2.1.1. Natural Products

The search for natural products comes from the reason that plants produce antimicrobials to protect themselves from exogenous organisms. It is thought that plants can produce anti-MDR compounds to fight the microbial resistance phenomenon. Carnosic acid (**1**) was one of the major products isolated from the plant *Rosmarinus officinalis*. When tested against the macrolide resistant strain of *S. aureus*, expressing the MsrA pump, compound 1 displayed favorable results, showing an impressive 8-fold potentiation of erythromycin [42].

An extract of *Lycopus europaeus* led to the isolation of two new isopimarane diterpenes, methyl-1 α -acetoxy-7 α ,14 α -dihydroxy-8,15-isopimarandien-18-oate (**2**) and methyl-1 α ,14 α - diacetoxy-7 α -hydroxy-8,15-isopimarandien-18-oate (**3**), which showed no antibacterial activity, but were able to reduce erythromycin's MIC from 256 μ g/ml to 128 μ g/ml, therefore causing a 2-fold potentiation. This behavior was similar to other isopimarane diterpenes isolated in this study, as well as for 5,9-dihydroxygeranylinalool (**4**) and 9-acetoxy-5-hydroxygeranylinalool (**5**), two diterpenoids [43-44].

Phytol (**6**) is an acyclic isoprenoid, and its EPI activity against the ABC pumps of *E. coli* was assessed in combination with nalidixic acid. Additionally, 15 derivatives were synthesized. One derivative in particular, compound 7, was able to decrease the MIC of nalidixic acid by 16-fold, and four other derivatives reduced the MIC by 8-fold. This effect was caused by the inhibition of ATP-dependent efflux pump and down-regulation of the efflux gene. *In silico* absorption, distribution, metabolism, and excretion (ADME) analysis was performed, taking into account parameters such as solubility in water, human intestinal absorption, plasma protein binding, blood-brain barrier penetration, hepatotoxicity and cytochrome P450 (CYP450) inhibition. Results showed all five active derivatives presented low

permeability into the central nervous system and displayed moderate intestinal absorption. In terms of plasma protein binding level, all five compounds exhibited levels below 90%, suggesting favorable distribution of the compounds into the blood. They also did not present hepatotoxicity, unlike reserpine, making them good lead compounds [45].

Ursolic acid (**8**) is a pentacyclic triterpenoid isolated from diverse natural sources. Its wide activity includes anti-inflammatory, antimicrobial, diuretic, and antitumor activity, among others. When tested against *Escherichia coli* strains that were resistant and susceptible to nalidixic acid, ursolic acid did not display intrinsic antibacterial activity. However, ursolic acid (**8**) was able to reduce the MIC of nalidixic acid by 2-fold, when both drugs were used in combination. This synergy led to further research on this scaffold. The first derivative prepared was the 3-*O*-acetyl ursolic acid, and showed a 4-fold decrease in the MIC of nalidixic acid. The lipophilic nature of this derivative, allied with the increased potency, led to the synthesis of other lipophilic ester derivatives. The methyl and ethyl esters of 3-*O*-acetyl ursolic acid did not show any increment in the activity. However, a further increase in the ester carbon chain increased the drug reversal potential by 2-fold, in comparison to 3-*O*-acetyl ursolic acid, against the resistant strain. This is the case of the isopropyl (**9**) and the *n*-butyl (**10**) derivatives. The addition of a pentyl group led to a decrease in activity. Furthermore, ursolic acid (**8**), and derivatives **9** and **10**, reduced the MIC of tetracycline by 2-fold, 8-fold, and 8-fold, respectively. When these compounds were tested against ATP-dependent efflux pumps, it was found that derivatives **9** and **10** were successful in inhibiting these pumps in terms of the liberated inorganic phosphate. Molecular modeling studies with the YojI pump, using maltose/maltodextrin transport ATP-binding protein as a homology model, showed that **9** was bound to the nucleophilic Ser-78, to the aromatic Phe-81, Trp-85 and Leu-86, the acidic Glu-94 and Glu-144 and the basic Ala-79, Ala-139, Ala-140, Ala-141, Ala-143, Arg-74, Arg-122, Arg-146, Leu-107, Val-80, Lys-96, and Ile-123 in the nucleotide binding domain. Derivative **10** was also subjected to the same kind of studies using the same model, and was predicted to bind in the same region [46].

A study with flavones and isoflavones from the plant *Sophora moorcroftiana* revealed that diosmetin (**11**) was able to inhibit three drug resistant strains of *S. aureus*, including the ABC family MsrA overexpressing strain, potentiating the effects of norfloxacin and streptomycin [47]. These results corroborate the works of Chan *et al* (2013), who had previously studied the effects of diosmetin (**11**) as an EPI [48]. This group observed a synergistic effect of this flavonoid with erythromycin, which was 5-fold greater than that of verapamil [48].

4-Hydroxy- α -tetralone (**12**) isolated from the plant *Ammannia multiflora*, showed enhancement against nalidixic acid resistant strains of *Escherichia coli*, prompting the synthesis of derivatives in order to infer their mechanism of action. Five derivatives were synthesized, and it was shown that compound **13** was able to inhibit the expression of the ABC transporter protein gene *yojI*, which encodes the ABC protein YojI, and was successful in combination with

tetracycline [49]. Molecular modelling studies with compound **13** and a homology model of the YojI protein revealed the following bound residues: Ser-78 (nucleophilic), Leu-86, Ile-123, Ala-137, Ala-140, Ala-141, and Ala-143 (hydrophobic), Glu-144 (acidic), Arg-122 (basic), Phe-81 (aromatic), Glu-144 (acidic), Arg-146 and Lys-96 (basic), and Trp-85 (hydrophobic), the same region as noted for ursolic acid derivatives **9** and **10**. Compound **13** showed good affinity and strong hydrophobic interaction with the YojI protein, corroborating the fact that this compound acts by the inhibition of ATP dependent efflux pumps [49].

The plant *Portulaca oleraceae* is used in folk medicine for its anti-inflammatory, anti-septic and diuretic effects, among others. A study of its fatty acids demonstrated that linoleic (**14**) and oleic acids (**15**) had synergistic activity with macrolides for the MsrA overexpressing strain of methicillin-resistant *S. aureus* (MRSA). In fact, erythromycin's activity was enhanced when in combination with either of these two compounds, likely through the inhibition of the MsrA ATP dependent pump. Since these fatty acids are essential for humans, low toxicity is expected, making them promising agents to use in combination with antibiotics [50].

Lysergol (**16**) is a clavine alkaloid of the ergoline family. Recently, its EPI activity was studied, and derivatives were synthesized [51]. The compounds were tested against susceptible and resistant to nalidixic acid strains of *E. coli* and a tetracycline resistant strain. While not possessing antibacterial activity, lysergol (**16**) showed synergy with nalidixic acid at the concentration of 10 μ g/ml, reducing its MIC by 8-fold in the resistant strain of *E. coli* and 4-fold in the susceptible strain. Of the thirteen semi-synthetic derivatives prepared, derivatives **17** and **18** reduced nalidixic acid's MIC by 8-fold, twice as much as lysergol (**16**), in the susceptible strain. A 4-fold potentiation of nalidixic acid was observed for the three compounds when tested against the resistant strain. As for the tetracycline resistant strain, all three compounds were able to reduce tetracycline's MIC by 8-fold. Further studies were performed, in order to conclude whether or not these compounds were capable of interfering with ATP-dependent efflux pumps, and it was proven that all three compounds inhibited ATPase activity, concerning the liberation of inorganic phosphate. A down-regulation of the gene *yojI* was also observed when the tetracycline resistant strain was treated with lysergol (**16**) and its two most potent derivatives, **17** and **18**, which further confirms the activity of these compounds as ABC transporter inhibitors [51].

2.1.2. Existing Drugs

As part of a screening program for P-gp inhibitors, GG918 (**19**) was discovered. This synthetic compound had already showed success in increasing the intracellular concentration of paclitaxel in cancer cells. In *S. aureus*, this compound was able to slightly potentiate the activity of norfloxacin in a strain overexpressing the ABC efflux pump

MsrA, suggesting that it may possess minimal activity over this pump [52].

The human P-gp inhibitors, vanadates, have been studied as inhibitors of the MutS pumps, belonging to the ABC ATPase superfamily. In fact, these compounds have demonstrated activity against the MutS in *P. aeruginosa* and *E. coli*. Decavanadate was the most effective, followed by orthovanadate (**20**), and they act non-competitively, possibly by replacing the inorganic phosphate. The fact that the ABC ATPases inhibited by orthovanadate are phosphorylated leads to the assumption that the low inhibitory effect could be attributed to the absence of a stable phosphorylation state of MutS [53].

3. MFS

The MFS is the most extensively studied family of transporters, present in both Gram-positive and Gram-negative bacteria, and comprises uniporters, symporters and antiporters. It also constitutes the largest family of secondary transporters, with over 10 000 sequenced members. Its substrates are diverse, and can range from ions, to carbohydrates, lipids, amino acids, nucleosides, among others. As many members of the MFS originate bacterial MDR, these transporters can pose as a good system for modulation studies [7, 19, 38, 54].

With the elucidation of the crystal structure of the MFS pump EmrD of *E. coli* (**Figure 4**, PDB 2GFP) [55], a general structure could be proposed for MFS transporters: twelve transmembrane helices that form a compact structure, with four of these transmembrane helices facing away from the interior cavity and the rest forming the internal cavity, constituted mostly by hydrophobic residues [7].

The efflux of drugs is only possible if the carrier alternates between two conformations, so that the substrate can bind to the convenient site in either side of the membrane. However, a limitation concerning the energy involved to displace the exposed hydrophilic surface of a protein can be inferred. It has been proved that either the oligomerization domain or the transport subunit provide the support needed to the movement of the substrate binding site, through a rigid-body rotation of the substrate binding domain, referred to as the "elevator mechanism" [56-57]. For this family, the most extensively studied efflux pump is the NorA pump of *S. aureus*.

The inhibitors that have proven activity against efflux pumps of the MFS family are gathered in **Figure 5**.

3.1. MFS inhibitors

3.1.1. Natural Products and Derivatives

Terpenes and terpenoids, existent in the essential oils of plants, have showed promising therapeutic potential in various fields. Therefore, studies about EPI potential of these compounds were carried out. Monoterpenes nerol (**21**), estragole (**22**), and dimethyl-octanol (**23**) were tested against

S. aureus. Compounds **21** and **23** were able to enhance the activity of norfloxacin, decreasing its MIC against the NorA overexpressing strain while not displaying intrinsic antibacterial activity. These open chain terpenes proved to be more effective than **22**, which carried a benzene ring, at inhibiting the NorA pumps [58].

Citronellal (**24**) and citral (**25**) are monoterpenes, and were chosen as starting material for the synthesis of new amide derivatives. The basis for this study was the fact that aromatic amides are recognized as potent EPIs, whereas alkyl, alkenyl, and alkynyl amides had not yet been explored. The compounds obtained were 5,9-dimethyldeca-2,4,8-trienoic acid amides and 5,9-dimethyldeca-2,8-dienoic acid amides, whose activity for the potentiation of ciprofloxacin in *S. aureus* was assessed. Of the first 22 synthesized compounds, seven were able to reduce ciprofloxacin's MIC by 4-fold. Further modifications were performed, and the C-9 methyl group was replaced by an amide, yielding compounds with similar potency as the previous ones. Derivatives subjected to hydrogenation led to decreased activity, which reveals that unsaturation is an important feature for antimicrobial potentiation. The mechanism of inhibition was assessed through ethidium bromide efflux, confirming these compounds act by inhibiting NorA, being compound **26**, a 9-formyl-5-methyldeca-2,4,8-trienoic acid amide, the most potent inhibitor [59].

Ferruginol (**27**), a diterpene, showed efficiency in inhibiting the efflux of ethidium bromide, proving its activity as a NorA inhibitor. It also showed a decrease in norfloxacin efflux in *S. aureus* [60]. Totarol (**28**), a phenolic diterpene, also showed a reduction in ethidium bromide efflux, suggesting activity against NorA pump, even though its mechanism is not yet known [61].

The triterpene ginsenoside 20(S)-Rh2 (**29**), a trace compound from red ginseng, also enhanced the effect of ciprofloxacin against *S. aureus*, through a NorA-mediated efflux inhibition, promoting the antibiotic accumulation in the bacteria [62].

Reserpine (**30**) is a natural alkaloid derived from *Rauwolfia vomitoria*, known as an anti-hypertensive agent, with inhibitory activity against human P-gp and breast cancer resistance protein (BCRP) [63-64]. Reserpine (**30**) has proven to be an effective inhibitor of the Bmr multidrug transporter in *Bacillus subtilis*, diminishing the efflux of ethidium bromide. Its structural analogue rescinnamine also had EPI activity, in a smaller extension than reserpine (**30**) [65]. Through DNA sequencing of the *bmr* genes of mutants, it was shown that reserpine (**30**) interacts with the residues Phe-143, Val-286, and Phe-306 of Bmr pumps, inhibiting drug transport [19]. Compound **30** has shown potential as an EPI for the inhibition of NorA efflux pumps in *S. aureus*, which are structurally related to Bmr pumps, reversing susceptibility to fluoroquinolones, demonstrating reductions up to 4-fold in MIC for some tested strains. It has been shown that **30**'s effect is more pronounced in hydrophilic fluoroquinolones, such as ciprofloxacin [66]. The mechanism by which **30** performs the inhibitory activity is through direct binding to the transporters that mediate drug

1
2 efflux, acting as a competitive inhibitor [19, 67]. Reserpine
3 (**30**) also succeeded in inhibiting the chloramphenicol efflux
4 by CmlR1 and CmlR2 of *Streptomyces coelicolor*, the
5 ciprofloxacin and norfloxacin efflux in *Listeria*
6 *monocytogenes*'s pump Lde, and the kanamycin and fusidic
7 acid efflux by LmrS from a MRSA clinical isolate. On the
8 other hand, for *L. monocytogenes*, **30** was not able to reverse
9 susceptibility to linezolid and lincomycin, which suggests
10 that **30** does not completely overlap with the structure
11 binding sites of efflux pumps, being the inhibition dependent
12 on the type and nature of the substrate [19]. However,
13 reserpine (**30**) shows neurotoxicity at the concentration
14 required to inhibit NorA, leading to the need to investigate
15 new inhibitors [67].

16 Piperine (**31**), an alkaloid found in the fruits of *Piper nigrum*,
17 was found to be an inhibitor of ciprofloxacin efflux in *S.*
18 *aureus*, reducing the MIC by 2-fold. It is thought that
19 compound **31** acts as a direct inhibitor of drug efflux.
20 Compound **31** inhibits drug transport of the pump MdeA,
21 potentiating the effects of benzalkonium chloride,
22 doxorubicin, daunorubicin, novobiocin,
23 tetraphenylphosphonium chloride, rhodamine 6G,
24 virginiamycin and mupirocin [17, 19-20]; it also inhibits
25 ciprofloxacin efflux by NorA [68].

26 Given these positive results obtained with piperine (**31**),
27 further research into this scaffold was performed. Kumar *et*
28 *al.* (2008) performed a screening of about 200 structurally
29 diverse compounds for the inhibition of NorA efflux pump,
30 choosing ethidium bromide as a substrate, since the only
31 mechanism through which this compound is expelled is
32 active transport (68). About 50 compounds were able to
33 increase the intracellular concentration of ethidium bromide
34 by 4-fold, while not displaying antibacterial activity. Five of
35 these compounds were more potent than piperine (**31**), being
36 three of these compounds selected for further studies: **32**, **33**,
37 and **34**, being **32** and **34** more potent than **31** by 40-fold [18,
38 68]. Assumptions regarding structure-activity relationship
39 (SAR) studies of these molecules could be drawn (**Figure 6**).
40 It was demonstrated that the introduction of an alkyl group at
41 C-4 position, mainly an isopropyl, isobutyl or di-isopropyl
42 group, coupled with the substitution of the piperidinyl
43 moiety with an aromatic amine, such as aniline, would lead
44 to maximum potentiation of ciprofloxacin; if the aniliny
45 moiety is substituted with a 2-hydroxymethyl group or a
46 nitrile, regardless of the positional isomery, ciprofloxacin
47 activity would increase. Replacing the piperidinyl moiety
48 with a phenylacetamide yields good potentiation of
49 ciprofloxacin, reducing its MIC by 4-fold, regardless of the
50 isomery. Unsaturation is crucial for the potentiation, as well
51 as the amide. Lastly, the retention of 3,4-
52 methylenedioxyphenyl or 4-methoxyphenyl groups in the
53 1,3-benzodiol moiety also gives favorable results [68-70].

54 Plants of the *Berberis* species have the ability to produce the
55 alkaloid berberine, which is a plant secondary metabolite
56 produced in response to microbial invasion, whose activity is
57 enhanced by a flavolignane also produced by these species,
58 5'-methoxyhydrnocarpin (**35**) [71]. Compound **35** exists in a
59 mixture of anion and neutral compound at physiological pH,
60

since it has a phenolic group with a pKa of 7.3. Due to the
acidic properties of this 7-OH group, **35** is likely to be a
specific microbial MDR inhibitor, as flavonoids with P-gp
inhibitor properties usually possess alkylated 7-O groups. In
fact, when added to a wild type strain of *S. aureus*,
compound **35** caused a decrease in norfloxacin's MIC to
0.25 µg/ml. Furthermore, when tested with berberine and
palmatine, **35** appeared to completely inhibit NorA,
suggesting it acts as a non-competitive inhibitor [71].

A number of hydrnocarpin-type flavolignanes were
synthesized in order to perform SAR studies. Flavolignanes
with and without phenolic groups at the 5 and 7 positions
had comparable activity. Diosmetin (**11**), the parent
compound, presents hydroxyl groups in the A ring and was
the most potent compound. Peracetate derivatives of **35** have
approximately the same potency as their parent compounds,
and could be considered prodrugs, if deacetylated by *S.*
aureus, which seems unlikely. Considering the D ring,
compounds bearing a 3''-methoxy-4''-hydroxy moiety
displayed good activity, and 3'',5''-dimethoxy-4''-hydroxy
moieties conferred a slightly less active compound. As for
the B ring, the presence of an H or an OH is not critical for
activity [72]. In addition, a flavone isolated from *Alkanna*
orientalis, sarothrin (**36**), blocked ethidium bromide efflux
by *S. aureus*, suggesting its potential activity as a NorA
inhibitor [73].

Biochanin A (**37**), an isoflavone, also showed potentiation of
norfloxacin and berberine against wild-type *S. aureus* and
Bacillus megaterium. An analogue of luteolin, orobol (**38**),
was quite active as a NorA inhibitor, unlike luteolin itself. It
was shown that flavones which have a single methoxy in its
B-ring are more potent than flavones with two methoxy
groups in the B-ring [74].

Another flavone, baicalein (**39**), was capable of potentiating
the effect of ciprofloxacin in *S. aureus*. Studies aimed at
measuring the efflux of ciprofloxacin and pefloxacin, a poor
NorA substrate, were performed, and showed that compound
39 was much more potent at restoring ciprofloxacin
sensitivity than pefloxacin, leading to the conclusion that this
flavone acts as a NorA efflux inhibitor, even though its
mechanism remains unknown [75].

Maia *et al.* (2011) identified six flavonoids from the plant
Praxelis clematidea: apigenine, genkwanin, 7,4'-
dimethylapigenin, trimethylapigenin, cirsmartin, and
tetramethylscutellarein (**40**) [76]. These methoxylated
flavones showed no antibacterial activity, but were able to
reduce the MIC of norfloxacin at least by 2-fold and up to
16-fold, as was the case of **40**, at a concentration of 8 µg/ml,
being therefore considered NorA efflux pump modulators. It
is thought that the lipophilicity of the flavonoids,
strengthened by the presence of methoxyl groups, is
responsible for this activity [21]. Presence of a methoxy in
the 4' position proved to be decisive for activity, as
flavonoids containing this group were more potent. The
number of methoxy groups is also essential for potency,
being the most methoxylated compounds the most active [76].
Tiliroside (**41**), an amphipathic kaempferol glycoside,
reduced the concentration of antibiotic needed to inhibit the

1
2 growth of bacteria. The lipophilicity of the flavone moiety
3 may be a determinant factor for the activity displayed.
4 Tiliroside (**41**) showed no antibacterial activity against *S.*
5 *aureus*, but showed a decrease in the MIC of
6 fluoroquinolones up to 16-fold, when at a concentration of
7 64 µg/ml. The reduction in the MIC of biocides, such as
8 ethidium bromide and benzalkonium chloride, was
9 impressive, with the MIC of acriflavine showing a decrease
10 by 128-fold at the concentration of 64 µg/ml and 32 µg/ml.
11 These results suggest that **41** might additionally act on
12 pumps other than NorA [77].

13 Kaempferol 3-*O*- α -L-(2, 4-bis-*E*-*p*-coumaroyl)rhamnoside
14 (**42**) was also able to potently inhibit in a concentration
15 dependent manner NorA-mediated ethidium bromide efflux
16 in *S. aureus*. This compound also increased the antibiotic
17 effect of norfloxacin against a wild-type and a NorA
18 overexpressing strain of *S. aureus*. However, it has to be
19 considered that **41** and **42** are flavonoid glycosides and
20 cinnamoyl esters, and that their activity can become
21 compromised with the activity small intestine β -glucosidases
22 or plasma esterases, even though the activity might possibly
23 lie in the kaempferol moiety, with **41** and **42** acting as
24 prodrugs. The authors hypothesized that if the activity of
25 compound **42** is compromised, this compound could be used
26 as adjuvant of ciprofloxacin for topical infections [78].

27 Since **42** showed promising results as a NorA EPI, the same
28 group performed a screening of a library of 117 chalcones,
29 due to their structural similarities with the coumaroyl
30 substituents [79]. Ten out of the 117 screened chalcones
31 presented medium efflux inhibition at 20 µg/ml. The most
32 potent chalcones, such as **43**, bear a dimethylaminoethoxy
33 moiety, which might disrupt the proton gradient, thus
34 eliminating the energy source of NorA. Five of these
35 chalcones possessed a hydroxyl group at the 4'-position, and
36 potentiated the activity of berberine against *S. aureus* and *B.*
37 *cereus*, suggesting that this substituent is also important for
38 activity. The compound with a methoxy group at position C-
39 2 has shown to present the best activity. The majority of
40 chalcones tested showed a 4-fold reduction of
41 ciprofloxacin's MIC in *S. aureus*, suggesting the presence of
42 a NorA efflux pump inhibitory activity. Compound **43**
43 showed 8-fold and 16-fold reduction of the MIC of
44 ciprofloxacin at 6.25 and 12.5 µg/ml, respectively. However,
45 it also showed antibacterial activity at 25 µg/ml. Some
46 chalcones also presented antiparasitic activity, namely
47 against *Leishmania major* and *Plasmodium yoelii* [79].

48 Another chalcone, 4',6'-dihydroxy-3',5'-dimethyl-2'-
49 methoxychalcone (**44**), showed good activity as a MDR,
50 causing complete growth inhibition at a very low
51 concentration (3.3 µg/ml) when combined with a sub
52 inhibitory concentration of berberine. When tested in
53 combination with berberine, erythromycin, and tetracycline
54 against strains of *S. aureus*, there was an increase in the
55 activity of all tested compounds in the wild-type *S. aureus*,
56 indicating a mode of action likely related to the NorA pump.
57 This compound was also tested against *B. cereus*,
58 potentiating the effects of the tested antibiotics as well, and
59 displaying a favorable effect, particularly in the case of

berberine, where a 30-fold increase in the activity was
observed [80].

Pheophorbide A (**45**) is an intermediate of the natural
breakdown of chlorophyll. Concentrations as low as 0.5
µg/ml of pheophorbide A with sub-inhibitory concentrations
of berberine were enough to completely inhibit *S. aureus*
growth. When extended to fluoroquinolones, this compound
also proved efficient in potentiating the effect of norfloxacin
by 4-fold in a wild-type strain, being its action directed
towards NorA [81].

Orizabins, oligosaccharides from resin glycosides, were
evaluated for their efflux pump inhibitory activity on strains
of *S. aureus*. These amphipathic compounds showed synergy
with norfloxacin, whilst showing no antibacterial activity by
themselves. Orizabin IX (**46**) completely inhibited the
growth of a NorA overexpressing strain at 1 µg/ml, while
orizabin XIX (**47**) reversed norfloxacin resistance by 4-fold
at 25 µg/ml for the same strain [82]. Murucoidins, which are
also resin glycosides, strongly potentiated the action of
norfloxacin against a *S. aureus* NorA overexpressing strain
by 4-fold at concentrations ranging from 5 to 25 µg/ml.
Stoloniferin I potentiated the same activity by 8-fold at a
concentration of 5 µg/ml [83].

A study aiming to find new EPIs from the plant *Geranium*
caespitosum led to the isolation of novel acylated
neohesperidosides. It was found that two compounds,
compound **48** and **49**, were successful at restoring
susceptibility to berberine, norfloxacin, and ciprofloxacin at
sub inhibitory concentrations, possibly due to NorA
inhibition [84].

Capsaicin (**50**), the major constituent of the fruits of the
Capsicum genus, is a known P-gp inhibitor. Studies were
performed, in order to evaluate if its inhibitory activity was
also applied to MDR pumps in bacteria. In a strain of *S.*
aureus overexpressing the NorA efflux pump, and therefore
resistant to fluoroquinolones, **50** proved efficient in
increasing the susceptibility to ciprofloxacin, while reducing
the emergence of ciprofloxacin-resistant mutants. Molecular
modelling studies of the complex capsaicin (**50**)-NorA, using
the glycerol-3-phosphate transporter to predict the structure
of NorA, showed that compound **50** aliphatic chain extended
into NorA's hydrophobic cleft. Furthermore, a weak
hydrogen bond between Arg-98 and the aryl hydroxyl
contributed to the stability of the complex [85].

Studies in the aqueous extract of *Artemisia absinthium*
showed promise in the inhibition of MDR efflux pumps. It
was proven that this activity came from caffeoylquinic acids.
At a concentration of 20 µM, 4'-5'-*O*-dicaffeoylquinic acid
(**51**) potentiated the activity of berberine by 16-fold and of
norfloxacin, by 10-fold against a wild-type strain of *S.*
aureus, while not displaying effects in a *norA*-deleted strain,
confirming this compound's NorA inhibition ability [86].

Olympicin A (**52**) is an acylphloroglucinol, found in the
aerial parts of *Hypericum olympicum*. This molecule had
some efflux impeding ability, proven by the improvement of
intracellular accumulation of enoxacin in a NorA
overexpressing strain of *S. aureus*, at 50 µg/ml [87].

Riparins are natural alkylamides found in the plant *Aniba riparia*. Since the extraction of these compounds comes with very low yields, synthetic approaches were developed. As such, the fundamental core of riparins, Rip-A (**53**), was synthesized and derivatives were prepared, Rip-B (**54**) to E (**55**). Rip-E (**55**) showed good growth inhibitory activity against *S. aureus*, attributed to their lipophilic nature and the presence of hydroxyl groups at the benzamide moiety. Oppositely, Rip-B (**54**), with two methoxy groups at the phenyl-ethyl moiety did not present antibacterial activity, presenting instead a decrease in the MIC of ciprofloxacin and norfloxacin, similarly to reserpine. Combined with the ability of decreasing ethidium bromide's MIC, the activity of compound **54** was attributed to NorA inhibition, suggesting its potential as an EPI [88].

Extracts of *Mirabilis jalapa* have shown good results in reversing fluoroquinolone resistance in strains of *S. aureus* overexpressing the NorA efflux pump. One compound in particular, *N-trans-feruloyl-4'-O-methyldopamine* (**56**), caused an 8-fold reduction in the MIC of norfloxacin, making it a potential lead in the search for structurally similar synthetic alkylamines [89].

Synthesis of derivatives was undertaken, taking *N-trans-feruloyl-4'-O-methyldopamine* (**56**) as a model. The aim was to identify active compounds from natural sources and, as such, couplings between cinnamic acid derivatives and natural-occurring amines were chosen. SAR criteria was taken into account, as it is known that substitution of the aromatic rings, methoxy or hydroxyl substitution, double bonds and aromatic ring nature in the amine part influence the activity. The lead compound was *N-trans-3,4-O-dimethylcaffeoyl dopamine* (**57**) since it showed the same activity as the natural compound. It was found that, for the cinnamic moiety, a hydroxyl substitution on the aromatic ring appears to be better than a methoxy group or unsubstituted derivatives and the double bond is essential for activity. As for the amine part, trisubstitution on the aromatic ring increases antibacterial activity, at the cost of decreased efflux pump inhibition. Methoxy substitution yields compounds with better results than hydroxyl substitution, which was better than no substitution and tryptamine combinations showed the best results (Figure 7). Among the compounds tested, compound **58** showed potentiation of norfloxacin comparable to that of reserpine (**30**). At a concentration of 30 μM , **58** showed good results for the inhibition of ethidium bromide efflux. For these compounds, it was shown that the activity reaches its highpoint when the phenyl ring is substituted with two hydroxyls [89].

Grapefruit oil fractionation led to the isolation of three compounds, two coumarin derivatives and one bergamottin derivative. The bergamottin epoxide (**59**) showed a reduction of ethidium bromide's MIC by 6-fold on different MRSA strains, and a 20-fold reduction in norfloxacin's MIC. This reduction was also achieved when the coumarin epoxide (**60**) was present, and suggests an activity towards NorA efflux pumps [90].

Coumarins isolated from *Mesua ferrea* were also studied for their efflux pump inhibitor activity against clinical strains

and NorA overexpressing strains of *S. aureus*. Seven coumarins were tested, and showed good results in inhibiting ethidium bromide efflux. Two compounds, **61** and **62**, showed potential EPI activity against the NorA overexpressing strain and a MRSA strain, using norfloxacin as a substrate, which makes them desirable compounds for the synthesis of derivatives [91].

Screening of natural products with P-gp inhibitory effects (92, 93) led to the identification of two compounds with NorA inhibitory activity, osthol (**63**) and curcumin (**64**) [36, 92-93]. Curcumin (**64**), derived from the rhizomes of *Curcuma longa*, has additionally already shown activity as an anti-inflammatory, antioxidant, antiviral, and anticancer agent [36, 92-93]. These compounds were effective in reducing the MIC of ciprofloxacin by 4- and 8-fold, respectively [94].

A penta-substituted pyridine, 2,6-dimethyl-4-phenylpyridine-3,5-dicarboxylic acid diethyl ester (**65**), was isolated from the rhizomes of *Jatropha elliptica*. This compound, when in association with ciprofloxacin, showed a strong effect against NorA efflux pumps in *S. aureus* [95].

A methanol extract of the plant *Dalea spinosa* yielded the isolation of six compounds, whose EPI potential was analyzed against wild-type *S. aureus* and mutant strains. It was shown that spinosan A (**66**), at a concentration of 48 $\mu\text{g/ml}$, and its acetate **67**, at a concentration 42 $\mu\text{g/ml}$, were able to decrease berberine's MIC by 8- and 62-fold, respectively, against the wild-type. Other compounds were also able to reduce berberine's MIC, but to a lesser extent. Compound **67** also managed to decrease berberine's MIC in the NorA overexpressing strain, suggesting inhibitory activity in this efflux pump [96].

3.1.2. Synthetic Compounds

The INF series (compounds **68-72**) resulted from an initial screening of 9600 structurally diverse compounds, in order to find NorA inhibitors for *B. subtilis*, not expressing Bmr. The NorA substrate chosen was ethidium bromide [67].

Since this screening did not focus on a specific chemical group, the most active compounds were divided into several groups. Several indole derivatives were active, which did not come as a surprise, since reserpine (**30**) itself has an indole moiety. Another large group was the trichloromethylamine containing compounds, which were not further explored as their likelihood to be toxic was high. Biphenyl urea derivatives were also active, and other compounds, with no obvious similarities, showed activity as well. Five compounds were selected for further tests, INF 55 (**68**), INF 240 (**69**), INF 271 (**70**), INF 277 (**71**) and INF 392 (**72**). These compounds were active at concentrations of 5 $\mu\text{g/ml}$ or less, and included the most potent compound, **72**, the most potent indole, **68**, and the most potent biphenyl urea, **71**. Derivative **72** was able to reduce resistance to ethidium bromide and ciprofloxacin by 8-fold at a concentration of 0.4 $\mu\text{g/ml}$. All five inhibitors were more potent than reserpine (**30**) at promoting the activity of ciprofloxacin in *S. aureus* and, by being structurally different, it was hypothesized that

1
2 a potent, nontoxic lead would be identified. These
3 compounds also proved effective in inhibiting the Bmr
4 multidrug transporter in *B. subtilis* and two compounds, **68**
5 and **71**, also enhance fluoroquinolone activity in
6 *Streptococcus pneumoniae* [67].

7 The results obtained with INF 55 (**68**) in *S. aureus* as an
8 inhibitor of the NorA efflux pump led to an increased
9 interest in this kind of scaffold. SAR studies regarding the C-
10 5 proved that substitution in this carbon is crucial for
11 activity. Substitution with a nitrile group leads to retention of
12 potency, and carbonyl based electron-withdrawing groups at
13 C-5 would result in molecules with no activity [97]. Three
14 dimensional-quantitative structure-activity relationship (3D-
15 QSAR) studies predicted that 2-aryl indole derivatives would
16 be NorA inhibitors. Functionalized INF 55 (**68**) showed a
17 decreased MIC for berberine, a MDR transporter substrate.
18 Berberine is an amphipathic cation alkaloid. From the series
19 of 2-aryl-5-nitroindoles synthesized by Samosorn *et al.*
20 (2009), the alcohol **73** was the most effective in inhibiting
21 berberine efflux, as well as other antibiotics, such as
22 ciprofloxacin, in *S. aureus* [98]. Later, the same group
23 developed a strategy that consisted in a molecule presenting
24 dual action: an antibiotic and a MDR inhibitor. Therefore,
25 they combined berberine, a hydrophobic cation that is a
26 MDR substrate, with INF 55 (**68**), a MFS inhibitor. These
27 efforts yielded compound **74** [17-18, 99]. In terms of
28 antibacterial activity in *S. aureus*, the hybrid was about 100
29 times more active than berberine alone, and the difference
30 was even higher when tested against a NorA overexpressing
31 strain of *S. aureus*. *Enterococcus faecalis* also presented
32 resistance to berberine, but was susceptible to **74**. [99]
33 Synthesis of sulfur derivatives was achieved based on the
34 premise that the activity of **68** is less dependent on the nitro
35 group than the 2-arylindole. Benzothiophene, thiophene, and
36 benzofuran derivatives, such as compounds **75**, **76**, and **77**,
37 respectively, were able to synergize with ciprofloxacin and
38 ethidium bromide, increasing their intracellular
39 concentration and leading to the conclusion that these
40 compounds inhibit NorA in *S. aureus*. [100]

41 Taking into account the fact that the indole NH is not
42 essential for efflux pump inhibition, Samosorn *et al.* (2009)
43 simplified the indole moiety of berberine-based hybrids,
44 along with the effect of a methylene ether linking group.
45 Their results showed that a hybrid molecule with oxygen in
46 the linkage chain had stronger antibacterial and MDR pump
47 inhibitory activity than the original **74** [101]. On the other
48 hand, removal of the indole moiety produced compounds
49 with lower dual activity, and this structural simplification led
50 to compounds with low antibacterial activity, but with
51 retained NorA pump inhibitory activity. These data suggest
52 that while the indole moiety is not necessary for NorA pump
53 inhibition, it is required for the antibacterial activity of
54 hybrid compounds [101].

55 A recent study identified a novel class of NorA EPIs with a
56 polysubstituted indole moiety. Twenty molecules were
57 highly active in inhibiting ethidium bromide efflux in *S.*
58 *aureus*. However, the best results were obtained with
59 compounds **78** and **79**, which were derivatives that presented

three and four carbon atom chains, respectively. Compound
78 presented the best half maximal inhibitory concentration
(IC₅₀), with a concentration of 1.8 μM. Compound **79**
exhibited similar IC₅₀ (2 μM) and ethidium bromide
inhibition as **78**. Compounds with shorter or longer carbon
chains did not present such good results, leading to the
conclusion that three or four carbons make up the right
distance between the indole scaffold and the basic center,
thus presenting the best inhibitory effect. When tested for
their synergy with ciprofloxacin, both **78** and **79** were able to
restore the antibiotic's activity [102]. It was found that the
substitution of the C-5 in the indole results in a potent EPI,
particularly if there is a propoxyl chain carrying terminal
cyclic amino groups, which appears to be crucial for NorA
inhibition at low concentrations. The presence of the *N*-
benzyl moiety preserves inhibition and contributes to the
modulation of the biological effects and ADME properties,
depending on its substituents [103].

Another successful example of a hybrid antibacterial/EPI is
the fluoroquinolone scaffold accommodating a bis-aryl urea
EPI moiety at the C-7 position. This kind of compounds was
successful in inhibiting NorA pumps in *S. aureus*.
Compound **80**, which incorporates a bis-aryl urea motif into
the ofloxacin core, proved to be a potent inhibitor of MFS
efflux pump systems, achieving 84 % inhibition rate at 10
μM. Moreover, these compounds also successfully inhibit
MepA pump systems from the MATE family [17-18, 104].
Fluoroquinolones containing a thiopyranopyridine moiety at
the C-7 position were synthesized and their activity was
tested against strains of *S. aureus*. However, these
derivatives did not seem to be NorA substrates. Further
investigations into thiopyranopyridinylquinolone esters were
carried out. Some of the synthesized compounds displayed
better inhibitory activity than reserpine (**30**), namely
quinolone esters **81**, **82** and **83**, and showed significant MIC
reduction of ciprofloxacin. Compounds **82** and **83** also
showed a 32-fold MIC reduction of ciprofloxacin for strains
of *S. aureus* overexpressing MepA pumps from the MATE
family [18, 105]. In terms of established SAR, these
compounds definitely do not need the fluorine atom at the C-
6 position for the antibacterial activity of
thiopyridinylquinolones. A methyl at the C-8 position yields
a compound with enhanced activity against Gram-positive
bacteria for 6-amino derivatives. The thiopyranopyridine
moiety at the C-7 position is crucial for NorA and MepA
inhibition (**Figure 8**) [105].

Derivatives of 2-(4-propoxyphenyl)quinolone were
synthesized based on the premise that its large hydrophobic
area and ability to establish an electrostatic interaction would
make them suitable inhibitors of NorA. These derivatives
also mimic the quinolone antibacterial core and possess a
versatile scaffold, making it prone to be very simply
chemically modified. These classes of compounds were
obtained by performing modifications in the 2-phenyl-4*H*-
chromen-4-one moiety, which is a common feature of
flavone and flavolignane EPIs [106].

Studies showed that the *O*-substituted 2-phenyl-4-
hydroxyquinoline derivatives displayed the best activity as

NorA inhibitors. Compounds **84** and **85** showed synergistic activity against a strain of *S. aureus* overexpressing NorA efflux pumps comparable to reserpine (**30**) and, comparing the results obtained with a mutant strain, absent of NorA, it was found that these compounds are able to completely restore the antibacterial activity of ciprofloxacin in the resistant strain [18, 106]. Preliminary SAR studies for this new class of EPIs showed that the best activity is when the quinolone core is replaced with the 2-phenyl-4-hydroxyquinoline group with an alkylation in the C-4 hydroxyl group. 2-Ethylamino chains inserted at the N-1 position of the quinolone nucleus or at the C-4 hydroxyl of the quinoline moiety provided compounds with better activity than those carrying the same chain at the C-2 of the phenyl ring. The best substituent for the C-2 phenyl ring appears to be the C-4' propoxy group (**Figure 9**) [106].

Taking these results into account, efforts have been placed to obtain more potent NorA inhibitors, introducing *O*-alkyl or different *O*-alkylamino chains at the C-4 position. The strategy was to determine the essential 3D structural requirements for the inhibition of NorA, based on the NorA EPIs described in literature through the use of computational methods, since the 3D structure of this pump was not elucidated yet. The results led to the building of a possible pharmacophore, which was used to design novel compounds, with different substituents at the C-4 position, which were synthesized and biologically evaluated [107]. The compounds were assayed for their ability to reduce the MIC of ciprofloxacin in *S. aureus* strains, and all compounds displayed this decrease for the strain overexpressing NorA pumps. One compound in particular, **86**, showed a 16-fold reduction in this strain for ciprofloxacin's MIC. SAR studies for this emerging class show that the best activities come from the compounds bearing the 2-ethylaminoalkyl chains linked to the C-4 hydroxyl group, such as compound **87**. The inclusion of an aliphatic ring including the side chain nitrogen atom led to retention of activity, whereas inclusion in an aromatic ring led to loss of activity, where the nitrogen nucleophilicity was reduced. The mono-substituted compounds, like **88**, with a benzyl group were well tolerated, and showed higher ethidium bromide inhibitory activity than compounds with two benzyl groups as substituents (**Figure 9**) [107].

3-(Dihydronaphthyl)-propenoic acid amines were evaluated for their EPI activity. Five compounds were synthesized, and two of them showed good results in inhibiting ciprofloxacin efflux in strains of wild-type and NorA overexpressing *S. aureus*. Further studies were performed, given the low EPI activity of this kind of compounds. Fifteen different compounds were synthesized, taking α -tetralone (**12**) as starting material. Out of these, four compounds showed significant MIC reductions for ciprofloxacin for the NorA overexpressing strain. One compound, **89**, even displayed a 16-fold reduction, while other remained in the range of 4- to 8-fold. These studies allowed SAR to be established. Addition of methoxy or allyloxy substituents at the 6,7-position of the 3,4-dihydronaphthalene leads to lower or no activity, whereas no substitution originated better EPIs.

Saturation of the double bonds lowered the potentiating activity (about 2-fold), while unsaturated derivatives made much more potent compounds [108].

Boronic species have been described for a variety of activities, namely antimicrobial and antineoplastic. Organoboron compounds, oxazaborolidines, boronic esters, and boronic acids are of particular interest, within this class. Fontaine *et al.* (2014) developed studies on these molecules, starting from a screening of a library of approximately 150 compounds, out of which 24 were hit compounds (exemplified with compounds **90** and **91**), and were able to restore the activity of ciprofloxacin by 4-fold at concentrations of 0.5 to 8 $\mu\text{g/ml}$ against the NorA overexpressing strain of *S. aureus* [109]. Preliminary SAR studies demonstrated the need of the boron atom for activity. Most pyridine-3-boronic acids showed best potentiating activity (e.g., compound **90**), whereas pyridine-4-boronic derivatives showed no activity, with exceptions, including a fluorinated ester. The shift of the boronic moiety from C-3 to C-4 led to a loss of activity and the trigonal boronic acid function gave better results than the sodium boronate salts and the potassium trifluoroborate. Concerning the pyridine-3-boronic acids, the most promising compounds appear to be the 6- and the 5,6-disubstituted ones, being the latter compounds less potent. Cyclisation at the C-5 and C-6 positions was well tolerated, as was the introduction of a methyl at the C-5 position. For the benzene analogues, which displayed good activity, cyclisation at the C-3 or C-4 was also well tolerated, as was the introduction of a methyl. A substituent at the *para* position to the boronic moiety proved to be very important. Two compounds were particularly efficient in inhibiting NorA, and did not display intrinsic antibacterial activity and cytotoxicity: the 6-benzyloxy pyridine-3-boronic acid **90**, which displayed the highest activity, potentiating ciprofloxacin by 4-fold at a concentration of 16 $\mu\text{g/ml}$, and the 4-benzyloxybenzene boronic acid **91** [109].

Compound **90**, being the most promising compound, was considered a hit, and its structure was used as a model for the synthesis of new derivatives. Therefore, the 6-benzyloxy group was substituted with (aryl)alkoxy chains with variable lengths (compounds **92** and **93** as examples), in an attempt to explore the putative hydrophobic binding site of NorA, and substituents were added to the phenyl ring. The ether linkage between the pyridine ring and the 6-benzyloxy group was substituted as well. The first substitution made was the replacement of the 6-benzyloxy group with alkoxy chains of diverse lengths, as to explore the size of the hydrophobic pocket of NorA. The potency of the compounds increased with the increase of the size of the side chain, although the compound with a 11-carbon side chain displayed no activity whatsoever. A new series of compounds with a phenyl ring at the end of the side chain were synthesized, with the aim of enhancing hydrophobicity, and the results showed these compounds were more efficient. It was concluded that the compound with the best performance possessed a side chain with seven carbon atoms and a phenyl group: compounds **92**, 6-(3-phenylpropoxy)pyridine-3-boronic acid, and **93**, 6-(4-

1 phenylbutoxy)pyridine-3-boronic acid, showed the highest
2 activity in the potentiation of ciprofloxacin (4-fold). The
3 introduction of substituents in the phenyl ring of compound
4 **90** led to unsatisfactory results, with the best compound
5 displaying moderate antibacterial activity. A methyl group in
6 the C-5 position did not show influence on the activity, and
7 the modification of the ether linkage led to varied effects:
8 introduction of a sulfanyl, thus conserving the H-bonding
9 acceptor capacity, led to similar potentiating activity,
10 whereas the introduction of an amine linkage, an H-bond
11 donor, led to a decrease in the activity. Finally, the shift
12 of the benzyloxy group from the C-6 to the C-5 resulted in no
13 activity [110].

14 Compounds AE-848/42434549 (**94**) and AN-465/42885978
15 (**95**) were hits in a virtual screening of novel NorA efflux
16 pump inhibitors in *S. aureus*. The structures deemed as
17 active *in silico* were later synthesized and evaluated on their
18 ability to prevent ethidium bromide efflux, as well as
19 ciprofloxacin potentiation. Compound **94** and **95** were as
20 potent as reserpine (**30**) for ethidium bromide efflux and
21 more potent at synergizing with ciprofloxacin. These hit
22 compounds **94** and **95** paved way for the synthesis of new
23 analogues, which allowed SAR studies to be made, in order
24 to assess which parts of their structure were determinant for
25 the inhibition of NorA. For compound **94**, it was shown that
26 limited modifications to the sulfone and amide group do not
27 cause significant changes in activity. On the other hand,
28 modifications to the imidazole ring result in a reduction of
29 activity. Replacement of the 1-methylpyrrole ring with a
30 furan also shows a decrease in the activity of the compound.
31 As for compound **95**, the *O*-benzyl group is essential and the
32 introduction of a benzyl in the secondary nitrogen leads to a
33 complete loss of activity. Furthermore, the methyl group of
34 the 2-(1-phenyl-1-propanol) moiety can be replaced with a 2-
35 (1-phenyl-1-ethanol) portion without a decrease in the
36 activity [111].

3.1.3. Existing Drugs

37 Several drugs already available for the treatment of various
38 diseases have shown potential to be used as MFS EPIs.
39 Reserpine (**30**), mentioned before, is one of them. Other
40 drugs include verapamil, omeprazole, paroxetine and
41 chlorpromazine. Even though bacterial MFS transporters and
42 mammalian ABC efflux systems, as P-gp, lack structural
43 homology, they share similar substrate profiles, which means
44 that mammalian MDR inhibitors can also present bacterial
45 efflux inhibition. However, the concentrations needed for
46 efflux pump inhibition are too high, which means that toxic
47 effects can arise if these compounds are used for this purpose
48 [18], [112]. In fact, the addition of verapamil to
49 chemotherapy was accompanied with cardiac arrhythmia and
50 hypotension [113]. This leads to the requirement of
51 developing analogues of these existing drugs maintaining the
52 desired EPI activity at concentrations that do not display
53 toxicity [112].

54 The antiarrhythmic verapamil, a known P-gp inhibitor, has
55 shown moderate NorA inhibitory activity in both *B. subtilis*

56 and *S. aureus*, enhancing fluoroquinolone activity [114-115].
57 However, the best results were obtained with proton pump
58 inhibitors omeprazole (**96**) and lansoprazole. Therefore, a
59 series of pyrrolo[1,2-*a*]quinoxaline derivatives mimicking
60 the omeprazole structure (**96**) were designed, with the aim of
61 evaluating their EPI activity against NorA. All the eleven
62 derivatives showed a reduction in norfloxacin's MIC, being
63 compound **97** the most active, reducing the MIC by 16-fold
64 at 128 µg/ml. These compounds did not show disturbance of
65 the electrical potential and the transmembrane pH, excluding
66 the alteration of the proton-dependent pump as the
67 mechanism of action. Instead, it is thought that these
68 compounds interact directly with NorA. It was shown that
69 the benzimidazole moiety is critical for the activity of these
70 compounds (**Figure 10**), and replacing it with an imidazole,
71 a pyridine or a pyrrolo[1,2-*a*]quinoxaline ring resulted in
72 loss of potency. Furthermore, if there is a methoxy-
73 substituent in the benzimidazole ring, potency increases.
74 Introduction of chlorine in the C-7 of the pyrroloquinoxaline
75 heterocycle provided an efficient restoration of norfloxacin's
76 bactericidal activity. Finally, the benzimidazolyl group was
77 also important for activity [112].

78 This was not the first time that pyrrolo[1,2-*a*]quinoxaline
79 derivatives were described as EPIs. In fact, a previous study
80 by the same group produced 12 new 4-[116]pyrrolo[1,2-*a*]
81 quinoxalines and one pyrrolo[1,2-*a*]thieno[3,2-*e*]pyrazine
82 compound. Two compounds, **98** and **99**, were more active
83 than reserpine (**30**), diminishing norfloxacin's MIC by 16-
84 fold in a NorA overexpressing strain of *S. aureus*. This study
85 showed that 4-[116]pyrrolo[1,2-*a*]quinoxalines and their 7-
86 methoxy analogues were more active, in contrast with the
87 chlorine- and 8-phenyl-substituted compounds, that
88 presented the lowest effects. Replacement of the *N,N*-
89 diethylamino group by pyrrolidine, its isomer with restricted
90 conformation, led to enhanced EPI activity, whereas
91 replacing it with a piperidine ring led to more flexible and,
92 consequently, less active compounds. The
93 pyrrolothienopyrazine **99** moiety yielded the best results,
94 suggesting that sulfur, an electron-rich atom, potentiates the
95 EPI activity [116].

96 GG918 (**19**) was discovered in a screening program aimed to
97 identify inhibitors of mammalian P-gp, and was able to
98 increase the concentrations of paclitaxel. This compound did
99 not present antibacterial activity against the strains of *S.*
100 *aureus* tested. However, the co-administration with
101 norfloxacin resulted in a 4-fold reduction in the MIC of the
102 NorA overexpressing strain [52].

103 Two mammalian MDR inhibitors, biricodar (**100**) and
104 timcodar (**101**), confer increased drug sensitivity to cells
105 expressing both the P-gp and multidrug resistance protein
106 (MRP)-1 efflux systems. They also show potentiation of
107 multiple antibiotics in Gram-positive bacteria. In fact, both
108 compounds were able to potentiate the activity of ethidium
109 bromide against *S. aureus*, *E. faecalis*, and *S. pneumoniae*.
110 For the specific case of *S. aureus*, both compounds were able
111 to partially reverse fluoroquinolone resistance. The fact that
112 the MIC of ethidium bromide was non-saturable for the three

bacteria tested suggests that **100** targets multiple efflux pumps [117].

A series of polysubstituted pyrroles was screened for their P-gp inhibition activity, which made way for the identification of a lead, and the compounds were further investigated for their bacterial efflux pump inhibition activity. A simplified pyrrole alkaloid, **102**, displayed dual activity as both a P-gp and a NorA inhibitor against *S. aureus*. This compound led to 4-fold improvement in the MIC of ethidium bromide in the NorA overexpressing strain. Compound **102** was also able to reduce ciprofloxacin's MIC by 8-fold when at 50 μ M [118].

Tariquidar (**103**), a third generation MDR modulator, with activity in inhibiting P-gp and BCRP efflux systems, has shown promise in inhibiting efflux pumps in *S. aureus*. In fact, this compound showed an increase of the isotopic form of ciprofloxacin in all investigated strains, displaying best results in the strain that overexpresses the NorA pump. [119]

The class of phenylpiperidine selective serotonin reuptake inhibitors (PSSRI), which includes the antidepressant paroxetine (**104**), showed efflux-related potential against *S. aureus*, particularly that conferred by NorA. In fact, paroxetine and its isomer, **105**, showed potency with respect to inhibition of this efflux pump against ethidium bromide.

The isomer **105** also interfered with the accumulation of norfloxacin by NorA, reducing by 4- to 8-fold its MIC. [120]

Femoxetine (**106**) is a paroxetine derivative, with activity as an EPI. [120] Studies were performed as to determine which moieties in paroxetine- and femoxetine-like PSSRI were detrimental for efflux pump inhibition in *S. aureus*, and conclusions from SAR are highlighted in **Figure 11**.

Synthesis of derivatives showed that the fluorine atom on the phenylpiperidine moiety is not associated with the activity. The *N*-substitution of the piperidine ring yields different kinds of activity, suggesting that the NH group forms a positive binding contact with the target pump, which does not happen if there is a *N*-substitution (compound **106**). The phenyl ether substituents also play an important role as determining the EPI activity. [121]

Also for this kind of compounds, a study was performed, in order to conclude whether the 4-phenyl ring is required for EPI activity and the effects of changes in the two-atom aryloxymethyl linker, replacing it with a thioether, amine, and alkene linker portions. Compounds **107** and **108** displayed the lowest MIC against *S. aureus*. Synthesis of multiple derivatives proved that the 4-phenyl moiety is not vital for EPI activity and that a variety of two-atom linker groups for the 3-aryl piperidine moiety can maintain EPI activity [122].

The ciclooxigenase-2 (COX-2) inhibitor celecoxib (**109**) has also received attention for its MDR inhibitory activity. There had been a link between COX-2 and *mdr1* gene expression, which has consequences in cancer chemo resistance, and the use of celecoxib (**109**) reversed drug resistance. If this is true for mammalian efflux pumps, for bacteria there is no evidence of a COX-2-like gene, suggesting that celecoxib acts differently in inhibiting MDR in bacteria. A study was carried out in order to prove this hypothesis, which

demonstrated that this drug helped in increasing sensitivity to antibiotics in *S. aureus* and *Mycobacterium smegmatis*. These effects probably occur due to the blockage of MDR transporters involved in the efflux of antibiotics, such as ampicillin, ciprofloxacin, kanamycin, and chloramphenicol. The observation of the accumulation of ethidium bromide intracellularly leads to the conclusion that this compound acts as a NorA inhibitor [123].

As celecoxib (**109**) demonstrated such promising activity, analogues were tested, which bore low or none anti-inflammatory activity and possessed the 1,4-dihydropyrazolo[4,3-*c*]-benzothiazine-5,5-dioxide nucleus. Ethidium bromide efflux inhibition was tested first *in silico*, and the 17 molecules that fit the chemical space requirements were then reduced to six, based on the activity prediction. These compounds were then screened for their *in vitro* NorA inhibitory activity, using an overexpressing strain of *S. aureus*. One derivative in particular, compound **110**, showed the best activity concerning ethidium bromide efflux (76.9%) which is better than celecoxib (**109**) (64.5%). When in combination with ciprofloxacin, **110** displayed results comparable to reserpine (**30**) and better than paroxetine (**104**), which makes it a lead compound for the development of inhibitors of this kind [124].

Phenothiazine and thioxanthene derivatives are currently used as neuroleptic and antiemetic agents, and have been described as modest, but broad, antibacterial agents. Although their antibacterial activity is not enough to be clinically relevant, they have shown synergy with standard antibiotic agents, and have also been associated with the inhibition of eukaryotic MDR efflux pumps, such as P-gp.

The mechanism through which these compounds exert their antimicrobial potentiation is not yet fully understood. However, it is thought that it might have to do with the inhibition of efflux pumps [125]. A study focusing on the activity of several compounds of these families against *S. aureus* was carried out, with the main aim of showing their potential in inhibiting NorA. The compounds tested, chlorpromazine, fluphenazine, thioridazine, prochlorperazine (**111**), *cis*(*Z*)-flupentixol, and *trans*(*E*)-flupentixol (**112**), displayed intrinsic antimicrobial activity and good results when ethidium bromide efflux was assayed against a NorA overexpressing strains of *S. aureus*. Additionally, phenothiazine **111** and thioxanthene **112** were also able to reduce the proton motor force of *S. aureus*, by reducing the transmembrane potential [125].

Since the phenothiazine moiety proved itself to work as a template for the synthesis of new MDR EPIs, it was chosen to be the scaffold for derivatives with improved activity. The logic for these studies lied in the elimination of the structural features that were responsible for neuroleptic activity. As such, drastic modifications were made, such as the elimination of one ring of the tricyclic benzothiazine backbone and of the chain linked to the N-10 atom, a tertiary amine whose protonation is crucial for the interaction with the dopaminergic receptor, and the addition of a substituted phenyl ring at the C-3 position, to guarantee better lipophilicity. Therefore, a prototype of new 3-phenyl-2*H*-

1
2 1,4-benzothiazines was developed. Preliminary screening on
3 the derivatives using a wild-type *S. aureus* strain, both with
4 and without ciprofloxacin, showed variable intrinsic and
5 synergistic activity. Two compounds, **113** and **114**, displayed
6 the best activity, while displaying no and weak antibacterial
7 activity, respectively. These compounds were able to inhibit
8 NorA efflux of ciprofloxacin, being suggested that **114** was
9 even able to completely inhibit this pump [126].

10 Thioridazine (**115**), a phenothiazine, was also tested for its
11 antibiotic potentiation, either in racemate or in its
12 enantiomeric forms. All the forms were able to reduce the
13 MIC of oxacillin in *S. aureus* and erythromycin in
14 *Streptococcus pyogenes*, being the L-enantiomer more
15 effective in the last case [127-128].

16 17 18 4. RND

19 RND pumps are found mostly in Gram-negative bacteria,
20 being the most responsible pumps for MDR in this kind of
21 organisms. They present a wide variety of lipophilic and
22 amphiphilic substrates, which include several classes of
23 antibiotics, antiseptics, dyes, and detergents. RND
24 transporters present a unique tripartite complex, constituted
25 by a minimum of twelve transmembrane segments: the
26 transmembrane pump, the outer membrane channel, and the
27 periplasmic adaptor protein, which puts the other two
28 components in contact. This structural organization makes
29 the extrusion of substrates directly into the external medium
30 possible [32, 38]. Even though it has always been thought
31 that RND transporters were exclusive to Gram-negative
32 bacteria, it has recently been identified and characterized
33 FarE in *S. aureus*, homolog to the AcrB protein in *E. coli*
34 [129-130].

35 Within this family, the most studied system is the AcrAB-
36 TolC, from *E. coli*. This efflux pump is composed by three
37 essential parts: a resistance-nodulation-division transporter,
38 AcrB; a membrane fusion protein, AcrA; and a
39 multifunctional outer membrane channel, TolC [7, 131-133].
40 It transports a wide variety of toxic compounds from the
41 intracellular space directly into the medium, evading the
42 periplasm, and using a proton gradient as the energy source
43 [132-133]. *Pseudomonas aeruginosa* also possesses an
44 efflux system of the RND family, the MexAB-OprM efflux
45 pump, which confers resistance to a wide variety of
46 antibiotics. This was the first and best characterized efflux
47 system in bacteria. It was found that AcrB (**Figure 12**, PDB
48 4ZLJ) is homolog to MexB (**Figure 12**, PDB 2V50), being
49 their structure very similar [7, 134].

50 **Figure 13** comprises all the RND pump inhibitors that will
51 be discussed in the following section.

52 53 4.1. RND inhibitors

54 4.1.1. Natural Products and Derivatives

55 One study revealed that the essential oil of *Helichrysum*
56 *italicum* can reduce the resistance to chloramphenicol in *E.*
57 *aerogenes*, *P. aeruginosa*, and *A. baumannii*. These data

suggested that one or more EPIs are present within the
essential oil since it was shown that geraniol (**116**) produced
significant restoration of sensitivity to chloramphenicol by
16-fold, and rendered a total sensitivity of the organism
when combined with of phenyl-arginine β -naphthylamide
(PA β N), which will be discussed further ahead [135].

A study published by Aparna *et al.* (2014) had the aim of
identifying natural compounds from plants that present
efficacy in potentiating effects of antibiotics in *P.*
aeruginosa overexpressing MexAB-OprM and *E. coli*
overexpressing AcrAB-TolC through *in silico* virtual
screening and pharmacophore approaches [136]. The
compounds with the best *in silico* results were then tested for
their EPI activity. The two compounds that showed the best
activity in potentiating the activity of carbenicillin and
levofloxacin in *P. aeruginosa* and *E. coli* were lanatoside C
(**117**) and daidzein (**118**). Lanatoside C (**117**) is cardiac
glycoside that inhibits the Na⁺-K⁺-ATPase, which can also
be the explanation for its EPI activity against MexB and
AcrB. Daidzein (**118**) is an isoflavone that has previously
showed slight EPI activity in *Mycobacterium smegmatis* and
as a modulator of P-glycoprotein, in human cervical
carcinoma KB-V1 cells [136].

An *in silico* study by Ohene-Agyei *et al.* (2014) identified
five phytochemicals that could be docked similarly to PA β N
in the binding pocket of AcrB. Of these five, three of them
were able to decrease resistance to antibiotics by inhibiting
the AcrAB-TolC system: plumbagin (**119**) increased
sensitivity to erythromycin, chloramphenicol, and
tetraphenylphosphonium; nordihydroguarectic acid (NDGA)
(**120**) potentiated the activity of erythromycin,
chloramphenicol, tetraphenylphosphonium, novobiocin, and
tetracycline; and shikonin (**121**) decreased resistance to
tetraphenylphosphonium [137].

A study proved the usefulness of curcumin (**64**) as a RND
EPI at a concentration of 50 μ g/ml, increasing the sensitivity
of carbenicillin, ceftazidime, and meropenem in *P.*
aeruginosa, in decreasing order. It also managed to
circumvent the resistance to gentamicin and ciprofloxacin in
resistant isolates, which PA β N failed, leading to the
assumption that curcumin inhibits efflux pumps differently
from PA β N [138].

A screening of 85 000 microbial fermentation extracts
derived from 3 600 strains of actinomycetes and 3 500
strains of fungi was made, with the aim of discovering EPIs
that increased sensitivity of levofloxacin in *P. aeruginosa*
overexpressing the MexAB-OprM or the MexEF-OprN
pump. Two compounds, EA-371 α (**122**) and EA-371- δ
(**123**), produced by a strain of *Streptomyces* sp.,
demonstrated inhibitory activity against MexAB-OprM
[139].

54 4.1.2. Synthetic Compounds

The first compound with potent inhibition of RND efflux
pumps was PA β N (**124**), a dipeptide amine. This dipeptide
was identified as a hit compound from the screening of 200
000 samples of small molecules that potentiate the activity of

antibacterial levofloxacin against strains of *P. aeruginosa* that overexpressed MexAB, MexCD and MexEF pumps [28, 140]. Compound **124** is a peptidomimetic, developed for using in adjunctive therapy. This compound is a substrate for RND pumps, acting as a competitive inhibitor of multidrug efflux systems, such as MexB, MexD, and MexF, binding to the substrate pocket, impeding antibiotic binding and/or extrusion. Particularly, this EPI binds in a location close to the antibiotic binding site, generating steric hindrance, making it more difficult for the antibiotic to bind. In other words, less quantity of antibiotic is exported, making its intracellular levels higher, thus restoring antibiotic sensitivity [11, 25, 27-28, 141-143]. However, **124** can affect the integrity of the membrane when used in high concentrations, making it prone to the emergency of resistant profiles, namely the modification of the lipopolysaccharide structure, leading to changes in drug penetration [24].

PA β N (**124**) has been validated against the AcrAB-TolC in *Klebsiella pneumonia*, *E. coli*, *Salmonella enterica* serovar Typhimurium and *Enterobacter aerogenes*, and in multiple homologous systems including *Acinetobacter baumannii*, *Campylobacter jejuni*, and *Campylobacter coli* [11, 142]. Its potency has been demonstrated against the norfloxacin resistance conferred by the Mex efflux system of *Pseudomonas aeruginosa*, the AcrAB efflux systems of the Enterobacteriaceae, and the erythromycin efflux system of *C. jejuni* [19].

Studies of SAR demonstrated that the basic middle amino acid, arginine, was detrimental for activity, although a substitution with L-lysine showed the same potency as the lead, and the introduction of L-ornithine, originating compound L-Phe-L-Orn- β -Na, showed a 2-fold increased potency. As the ornithine moiety provided a simpler synthesis, it became the standard middle residue in successive compounds [25, 142, 144]. However, these compounds were not stable upon incubation with human serum, due to the occurrence of cleavage of peptide linkage between both amino acids, which is explained by the fact that natural (L) amino acids are contained within its structure. In order to overcome this problem, methylation of the NH that links both amino acids was achieved, maintaining potency and being this derivative able to resist serum proteases. Serum stability was also increased by replacing the L-amino acid with D-amino acid [25, 142]. In the same study, the authors also proved that the amino acid in the *aal* position (**Figure 13**, **124**) should be appropriately substituted, and the replacement of phenylalanine with a homophenylalanine led to improved potency. The problem with this compound was the fact that the ornithine moiety could easily form a lactam, rendering the compound inactive. This was overcome by switching the L-ornithine with the L-phenylalanine, which originated L-Orn-L-hPhe- β -Na, which originated a compound just as potent, but with no propensity to form a lactam. As for the β -aminonaphthalene moiety, it was proven that replacing it with 3-aminoquinoline led to reduced cytotoxicity and intrinsic antibacterial activity, even though its potency was slightly reduced [25, 142]. These efforts originated the compound L-Orn-L-hPhe-3-NHQ

(**125**). In conclusion, the analogues synthesized by Renau *et al.* (1999) did not show improvements in potency when compared to PA β N (**124**), but showed increased stability [142].

The same group developed further studies with this type of molecules and demonstrated that conformational restriction of the amino group of ornithine showed less toxicity, while maintaining potency. Such compounds were attained by adding a proline substituent, thus incorporating the two basic residues necessary for activity, which resulted in the successful synthesis of constrained derivatives, more potent and less toxic than **125**. The most interesting derivative, compound **126**, was as potent as **125**, but more than 4-fold less toxic. This compound also retained the activity against RND efflux pumps MexAB-OprM, MexCD-OprJ and MexEF-OprN. Although this compound showed similar protein binding in rats as **125**, its pharmacokinetic parameters were improved, and its efficacy in combination with levofloxacin was demonstrated in an *in vivo* model [145].

In a latter study developed by the same group, several non-peptide analogues were synthesized, and it was hypothesized that the peptide backbone is not essential for the activity of this type of compounds. The results suggested that the inhibition of pumps was possible due to the di-cationic nature of the compound, the appropriate lipophilicity and a disposition similar to that of compound **125** [146].

MBX2319 (**127**) is a pyranopyrimidine EPI inhibitor of AcrAB-TolC efflux pump in *E. coli* and other Enterobacteriaceae [141]. Compound **127** did not show any antibacterial activity, which is a positive feature for EPIs. It also potentiated the antibacterial activity of fluoroquinolones, such as ciprofloxacin and levofloxacin, and β -lactams, such as piperacillin against strains of *E. coli* that presented AcrAB-TolC efflux pumps [28, 141]. In *P. aeruginosa*, although compound **127** presents activity against RND-type pumps, it is unable to penetrate the outer membrane, which is highly selective [28].

In *E. coli*, the target of compound **127** is the membrane transporter AcrB. This molecule binds to the hydrophobic trap of the T protomer, interacting with the hydrophobic residues that constitute the deep binding pocket as a hydrophobic trap. π - π Interactions also occurs, between the pyridine ring of **127** and the aromatic side chain of Phe-628. The phenyl and morpholinyl groups also interact with Phe-178 and Phe-615. The dimethylenesulfide linker and the geminal dimethyl group present van der Waals interactions between the side chain of Phe-178, and Tyr-327 and Met-573, respectively [28, 147].

SAR studies demonstrated that the maintenance of activity depended on the geminal dimethyl moiety of the tetrahydropyran, the nitrile group and the length of the dimethylenesulfide linker. This data is presented in **Figure 14**. On the other hand, the morpholine moiety and aryl group are both prone to substitution, originating more stable derivatives in terms of activity, solubility, and metabolism [28]. Studies with this scaffold have shown that non-acidic substituents can be added to the phenyl group, in order to

1
2 improve potency and CYP450 inhibition, and modifications
3 to the morpholinyl group has greater effects on solubility and
4 stability [140].

5 Analogues were synthesized by Sjuts *et al.* (2016), by
6 changing the structure and functional groups of compound
7 **127** (148). They reached the conclusions that the
8 introduction of a 2,6-dimethyl to the morpholinyl group
9 improved both microsomal stability and EPI activity. The
10 replacement of the morpholinyl group with 2-
11 methoxyethylpiperazinyl improved aqueous solubility, at the
12 cost of a slight reduction of EPI activity. Two analogues, **128**
13 and **129**, resultant of the combination of 2,6-
14 dimethylmorpholinyl and acetamide or acrylamide,
15 respectively, on the phenyl group, resulted in a 10-fold to 20-
16 fold increase in EPI activity against *E. coli*, respectively,
17 comparing to MBX2319 (**127**) [28, 148]. The increased EPI
18 activity of these compounds has its basis in the acetamide
19 and acrylamide groups and the morpholinyl group. The
20 acetamide groups are engaged in highly ordered and
21 complex hydrogen bonds, with its center in a solvent water
22 molecule, which plays the role of a hydrogen bond donor to
23 the carbonyl backbone oxygen of Ala-286 and Gln-151 side
24 chain of AcrB. The acrylamide group forms a bridging
25 hydrogen bond through a water molecule to the side chain of
26 Gln-176 [28].

27 Nguyen *et al.* (2015) also synthesized a series of analogues,
28 with the morpholine scaffold substituted by a 2,6-
29 dimethylmorpholinyl group (140). Many of these analogues
30 exhibited a solubility improvement of 10-fold, compared to
31 compound **127**. The most potent and promising compounds
32 of these series were compounds **130**, **131**, **132**, **133** and **134**
33 [140].

34 Through a screening of an *N*-heterocyclic organic compound
35 library, Bohnert and Kern (2005) were able to find a novel
36 class of EPIs, the arylpiperazines [25, 149]. The aim of their
37 work was to find compounds that would potentiate
38 levofloxacin potency against strains of *E. coli*
39 overexpressing *acrAB* and *acrEF*. This was fulfilled when
40 they found phenylpiperazine derivatives with promising
41 activity [25, 149]. SAR studies suggested that elongation of
42 the spacer between the benzene ring and the piperazine ring
43 would enhance potency. Also, substitutions on the benzene
44 ring by halogen atoms led to an increase in potency [24,
45 149]. On the other hand, the potency would decrease when
46 an ethyl or phenyl group was added to the piperazine ring.
47 With this, the authors found the most potent
48 phenylpiperazine, *meta*-TFMPP (**135**), and
49 naphthylpiperazine, 1-(1-naphthylmethyl)-piperazine (NMP)
50 (**136**) [149].

51 NMP (**136**) was the most potent unsubstituted
52 arylpiperazine, able to increase the intracellular
53 concentration of chloramphenicol, tetracycline, linezolid,
54 fluoroquinolones, and macrolides. Although it does not
55 display action in *P. aeruginosa*, it shows effectiveness in *A.*
56 *baumanii* and several Enterobacteriaceae [11, 23]. EPI
57 activity was also reported in clinical isolates of *E. coli*, most
58 relevantly in fluoroquinolone resistance [150]. Through
59 computational analysis, it was found that NMP (**136**) binds

with high affinity to the lower part of the deep pocket of the
B monomer, overlapping in part the binding site of
chloramphenicol [11]. The binding site for NMP (**136**)
includes interactions with hydrophobic residues near the
hydrophobic patch and Gly-617 of the G-loop. This data
suggests that NMP (**136**) interferes with the movement of the
G-loop, important for extruding substrates, thus inhibiting
the action of AcrB [25].

Nakayama *et al.* (2003) performed a series of syntheses in
order to attain a compound specific for the inhibition of a
MexAB-OprM specific EPI [151-152]. They identified a hit,
compound **137**, through high-throughput screening for
levofloxacin potentiation using a strain of *P. aeruginosa*
strain overexpressing MexAB-OprM. The features this group
was looking for was the effective inhibition of efflux pump
and the lack of intrinsic antibacterial activity, since efflux
pumps are not vital for the organism's survival [151-152]. It
was shown that this compound effectively potentiated the
activity of levofloxacin, leading to an 8-fold decrease in the
antibiotic's MIC. However, its physicochemical properties
were problematic: water solubility was poor and it presented
high affinity to serum albumin. Therefore, they divided the
molecule into three parts, A, B, and C (**137**) and looked for
alternative scaffolds and substituted polar groups, in order to
reduce serum protein binding [152].

It was shown that when a carboxamide was introduced in A,
the affinity to serum albumin was diminished, as was its
activity. More polar moieties led to a complete loss of
activity. The strategy for the styrene portion (B) that was the
most successful was to substitute the benzene ring to a 3,5-
disubstituted pyridine, which had both good activity and no
major serum influence. As for the vinyl group in B, it was
demonstrated that molecules bearing methoxy and ethylene
moieties displayed favorable activity profiles. As for part C,
the basis of the modifications was the fact that the
introduction of bulky or hydrophilic groups adjacent to the
carboxyl group would reduce protein binding. An
enantiomeric pair containing a dioxolane moiety presented
both activity and serum stability, being the *S* enantiomer
more active [152].

Regarding the general scaffold, new compounds were
designed, keeping in mind that the thiazole moiety was
crucial for activity. Therefore, a molecule having a thiazole
side chain attached to the C-7 position of a quinolone was
synthesized. Substitution with a tetrazole portion yielded
better results than substitution with a carboxylic acid. One
compound (**138**) presented the best activity and serum
stability [153].

Further efforts were placed in order to increase the potency
of this compound. The *in vitro* activity was enhanced by the
introduction of a hydrophobic group at the 2-position of the
pyridopyrimidine scaffold, and hydrophilic substitution
would not compromise the activity. The most promising
compound had the inclusion of an olefin spacer between the
tetrazole and the pyridopyrimidine scaffold, with the
limitation of being prone to photoisomerization [154]. To
circumvent this, the ethylene tether between the
pyridopyrimidine was replaced with an amide bond,

1
2 providing stability against photoisomerization and,
3 unexpectedly, improving potency. However, another
4 problem arose: the lack of solubility for intravenous use
5 [155]. The following step was the introduction of a
6 secondary amine, giving a zwitterionic analogue, with
7 retention of activity. C-2 Analogues, namely with cyclic
8 substituents, also showed promising results [156]. Once
9 again, the problem of solubility arose. Therefore, efforts
10 were placed in making the molecule more hydrophilic, with
11 the introduction of substituted aromatic moieties. Analogues
12 4-substituted were the most potent, and the introduction of a
13 morpholine moiety led to increased solubility, maintaining
14 the serum stability. Compound **139** was the most balanced
15 molecule in terms of the desired features [157]. The last
16 refinement made was the introduction of a quaternary
17 ammonium salt, which led to D13-9001 (**140**). Adding to its
18 high solubility and safety, it was able to potentiate the
19 activity of aztreonam [158]. **Figure 15** illustrates the SAR
20 established for **140** and derivatives.

21 3D Crystal structures of **140** bound to the target, either AcrB
22 or MexB, show that the *tert*-butyl thiazolyl aminocarboxyl
23 pyridopyrimidine moiety of this compound binds tightly to a
24 narrow depression, designated by hydrophobic trap, close to
25 the deep substrate binding pocket. Additionally, the tetrazole
26 ring and the piperidine acetoamino ethylene ammonio-
27 acetate moiety interact with ionic and/or hydrophilic residues
28 in the substrate translocation channel. The piperidine
29 acetoamino ethylene ammonio-acetate moiety also covers
30 part of the minocycline and doxorubicin binding site [23, 28,
31 151].

32 Quinoline derivatives with EPI activity include a multiplicity
33 of derivatives, including pyrrodo-, alkoxy-, thioalkoxy-,
34 alkylamino- and chloro- derivatives. These compounds were
35 able to restore an intracellular concentration of antibiotic
36 drugs expelled by efflux pumps, as well as to induce the
37 increase in antibiotic susceptibility, acting as competitive
38 inhibitors of the antibiotic flux. In *E. aerogenes* and *K.*
39 *pneumoniae*, quinolines were more effective than PA β N
40 (**124**) in inhibiting the activity of the AcrB pump,
41 potentiating the activity of fluoroquinolones, tetracycline,
42 and chloramphenicol [11, 24, 26]. Among the different
43 classes of substituted quinolines, it was proven that the best
44 side chain was piperidinoethyl, which potentiated efficiently
45 the restoration of drug susceptibility in alkoxy- and
46 thioalkoxyquinolines. The heteroatom connecting the side
47 chain is also crucial for activity, being the most potent the
48 amino substituent, then the thioalkyl-, followed by the oxo-
49 derivative. The position of the branched substituted groups is
50 also detrimental for the EPI activity [24]. Branched side
51 chains containing piperidinoethyl and morpholinopropyl
52 groups were associated with the most interesting results,
53 being compound **141**, containing a piperidinoethyl side
54 chain, the most active when in combination with
55 chloramphenicol [159]. It has been hypothesized that the
56 pump inhibition is greatly influenced by the branched side
57 chain, and takes place either in the inner-membrane
58 transporter or at the junction between the inner pump and the
59 outer channel [159].

Given these promising results, further research in this kind of
compounds was performed. Alkoxyquinolines were
synthesized and tested against a strain of *E. aerogenes*
overexpressing the AcrAB efflux pump. Compound **142**
induced an increase in the susceptibility of chloramphenicol,
tetracycline, and fluoroquinolone. This could happen due to
interference during active pumping out of the antibiotic
[160].

Chloroquinolines also demonstrated efficacy as EPIs,
modulating chloramphenicol activity, decreasing its MIC 8-
fold for resistant strains. Synthesis of analogues led to the
conclusion that amino derivatives are more potent than thiol
derivatives, pointing out the importance of the side chain for
the efflux inhibition. Protonation of the nitrogen could also
play an important role at environmental pH, stimulating
recognition and binding of the drug to the sites located
within the pump's cavity. The heteroatom is also crucial for
the activity, being nitrogen the most efficient. Compound
143 is the chloroquinoline with the best results found for the
inhibition of the main efflux pump of *E. aerogenes* [161].

Alkylaminoquinazoline derivatives lacking a nitro group
were also evaluated for their ability to decrease
chloramphenicol and quinolone efflux in *E. aerogenes*,
through the inhibition of AcrAB efflux pump. The results
obtained suggest that this family of compounds recognizes
the same pump site responsible for the transport of
chloramphenicol and quinolones, competing with them.
Structurally, it has been shown that a morpholine group with
a propyl chain makes a more active alkylaminoquinazoline.
The most active compound of this family is compound **144**
[162].

Benzothiazoles 2-substituted were first tested for the
potentiation of ciprofloxacin in AdeABC overexpressing *A.*
baumanii. These compounds did not show antibacterial
activity when tested alone in bacteria. However, when
combined with ciprofloxacin, there was an observation of a
reversal in antibacterial susceptibility. The synthesis of
several derivatives of this family led to pharmacophore
studies, regarding the features of the most promising
compounds. Using computational methods, it was revealed
that, in order for the compounds to be biologically active, the
following features should be present: the nitrogen atom in
the thiazole ring and the carbonyl oxygen in the amide
function substituted on the second position of the
benzothiazole ring must have an hydrogen bonding acceptor
property; the benzene ring in the fused ring system, the
phenyl group attached to the second position of the
benzothiazole ring and the phenyl ring in the 2-
phenylacetamide and/or 3-phenylpropionamide moiety are
essential, since they provide a hydrophobic aromatic
property. Of the synthesized compounds, the ones that
gathered these features and, therefore, presented the most
promising activity, were compounds **145**, **146**, and **147**
[163]. These compounds were also tested for their activity
against AcrAB-TolC, in an overexpressing strain of *E. coli*,
reducing the MIC of ciprofloxacin 10-fold, in the case of **146**
and **147**, and 8-fold, for **145** [164]. Unlike AdeABC,
AcrAB-TolC's crystal structure is elucidated, making

docking studies possible. This predicted that all three compounds act as AcrB substrates, binding to the distal pocket site in the AcrB porter domain by blocking or inhibiting the ciprofloxacin binding site, generating steric hindrance and thus impeding the binding of the antibiotic. Furthermore, **146** and **147** showed stronger binding interactions than ciprofloxacin [164].

Indole derivatives have been useful in inhibiting protein targets of virus, tumors and bacteria. Since small molecules with conjugated aromatic rings, in which indoles fit, present high potential as EPIs, efforts were placed in synthesizing derivatives that presented this kind of activity. The TolC 3D structure has already been disclosed, making possible the structure-based design of inhibitors. Indoles **148** and **149** were proven efficient inhibitors of TolC in *E. coli*, potentiating the effects of chloramphenicol, tetracycline, erythromycin, and ciprofloxacin, with decreased MIC values of 2-fold for tetracycline and erythromycin and 8-fold for the other two [165].

4.1.3. Existing Drugs

Other drugs used for treatments other than antibacterial have demonstrated EPI activity. Tetracycline resistance in *P. aeruginosa* was reduced 8-fold when the phenothiazine fluphenazine, an antipsychotic, was present. Chlorpromazine (**150**) also showed EPI-like activity for the BpeAB-OprB system in *Burkholderia pseudomallei* and a reduction of the expression of *acrB* in *E. coli* and *S. enterica* serovar Typhimurium, suggesting an inhibition of AcrB production [11, 27, 128].

Selective serotonin-reuptake inhibitors have been studied for their ability to act as EPIs for RND efflux pumps in *E. coli*. It was found that sertraline (**151**) was capable of potentiating the activity of fluoroquinolones. However, its activity is limited, since it is possible that this compound also acts as an inducer of efflux pumps. Studies have shown that this compound could accumulate in tissues or cells, and therefore, sertraline's (**151**) concentration may be higher than that measured in plasma. This means that a standard dose of this drug could enhance antibiotic efficacy. It has not yet been studied if a sertraline (**151**) metabolite could be administered at a dose higher than sertraline. However, it was hypothesized that it could have a better EPI activity, while causing less undesired effects on the central nervous system [166].

Through the observation of previously reported EPIs, Piddock *et al.* (2010) suggested that simple heterocyclic nitrogen-containing compounds could also act as EPIs and synergize with ciprofloxacin in strains of *S. enterica* serovar Typhimurium that overexpressed the AcrAB-TolC efflux pump [167]. It has been shown that compounds containing a phenylethylamine moiety, as PAβN (**126**) does, such as epinephrine, norepinephrine, and cathinone, are able to potentiate the activity of ciprofloxacin. Of the compounds tested, trimethoprim (**152**) and epinephrine (**153**), showed the best results for the tested strains, which included, apart from *S. enterica* serovar Typhimurium, *P. aeruginosa*, *E.*

coli, and *K. pneumoniae*. These compounds showed synergy for the strains where AcrAB-TolC was active, but showed no activity when the pump was inactive. Thus, it can be hypothesized that these compounds either inhibit the expression of the genes encoding AcrAB-TolC, or interact directly with the pump [167]. Both compounds possess structural features similar to previously described EPIs, such as PAβN (**124**), L-Orn-L-hPhe-3-NHQ (**125**), and NMP (**136**), such as an aromatic ring linked to a basic nitrogen, in the case of epinephrine (**153**), or a pyrimidine ring, for trimethoprim (**152**). Trimethoprim (**152**) is already used in combination with another antibiotic, sulfamethoxazole, and combination with a quinolone was hypothesized to be a viable choice. On the other hand, a combination of epinephrine (**153**) with an antibiotic does not seem a viable option, because of the likelihood of the occurrence of sympathomimetic effects [167].

The antimalarial artesunate (**154**) has also shown EPI activity, enhancing the effects of β-lactam antibiotics, such as penicillin G, oxacillin, and ampicillin, and novobiocin against *E. coli*. The mechanism through which **154** expresses its activity is by the inhibition of the expression of the AcrAB-TolC system, not exhibiting antibiotic activity by itself [168].

5. SMR

The SMR family comprises the smallest drug efflux proteins known, possessing only four transmembrane segments in its composition, and are exclusive to bacteria. They are involved in the efflux of lipophilic compounds, such as quaternary ammonium salts and a variety of antibiotics. These pumps also use an electrochemical proton gradient in order to exert its efflux activity [7, 32, 38, 169].

One example of a SMR-type pump is the EmrE transporter (**Figure 16**, PDB 3B61), present in *E. coli*. In the absence of the ligand, the asymmetric unit of EmrE contains eight EmrE monomers, with four transmembrane helices composing each of them. If a ligand is present, it can function as an inverted homodimer [7, 170].

There have not been many studies concerning inhibitors of this family of efflux pumps, and the few that have been performed were only *in silico*. **Figure 17** shows the structures of the SMR inhibitors that will be further discussed.

5.1. SMR inhibitors

5.1.1. Natural Products

Quercetin (**155**) is a flavonol, present in many plants. This compound is very well tolerated in humans, as are its glycoconjugates, and presents a multiplicity of activities, such as antioxidant, anti-inflammatory and antimycobacterial. Only docking studies of this compound with two pumps belonging to the SMR family, the Mmr, in *Mycobacterium smegmatis*, and the EmrE pumps, in *E. coli*, were carried out. In Mmr, the hydroxyl groups present in the

3' and 4' positions of the B ring of quercetin were predicted to interact with Glu-14 in the helix 1, and the keto group of the hydroxychromen ring of the ligand interacts with the Trp-48 of the helix 1. Additionally, hydrophobic interactions were shown by the residues Phe-44, Tyr-40 and Trp-48, and π - π interactions were found with Phe-44. Furthermore, **155** was also predicted of interacting with dimeric forms of the protein [171]. In the case of EmrE, the interaction between the 3' and 4' hydroxyls with Glu-14 can also be observed, as can the hydroxyl in the C-7 position with Tyr-60. Hydrophobic interactions are present, with the residues Ile-11, Ser-64, Gly-67 and Leu-70, and π - π interactions exist with the residues Tyr-60 and Trp-63. Structural studies suggest that EmrE is mostly in the dimer state, which also interacts with quercetin (**155**) [171].

5.1.2. Synthetic Compounds

Cation ligands were subjected to docking studies with the Mmr protein of the SMR family in *Mycobacterium tuberculosis* and the EmrE dimer from *E. coli*. The rationale behind this study is the fact that SMR can extrude cations. This study led to the identification of new virtual leads, amongst which is compound **156**. ADME studies prove that all the molecules present in this study obey the Lipinski rule of five and the Jorgenson rule of three, which shows that the compounds possess good drug-like properties [172].

6. MATE

MATE transporters are responsible for the efflux of cationic, lipophilic substances, providing bacteria and cancer cells with MDR, using Na⁺ or H⁺ influx. They have 12 transmembrane helices, constituted by 400 to 700 amino acids. The mechanism of efflux is thought to happen by a rocker-switch mechanism. Members of the MATE family include the MepA (**Figure 18**, PDB 1TZP) transporter, in *S. aureus*, even though seventeen MATE proteins have been identified in eleven species, capable of extruding antibiotics. Ethidium bromide, tetraphenylphosphonium, acriflavine, berberine, and norfloxacin have been reported as MATE substrates [38, 173-181].

In **Figure 19** are compiled the structures of the compounds that have so far proven their usefulness as MATE inhibitors.

6.1. MATE inhibitors

6.1.1. Natural Products

A study performed by Kesharwani *et al.* (2017) accomplished the docking of phytochemicals with the NorM transporter, belonging to the MATE family [182]. After a screening of a library of natural products, three hits were identified: derhamnosyl suspenaside (**157**), prunin 7''-O-gallate (**158**), and quercetin diglucoside (**159**). These were the best compounds, as their results in binding free energy calculation were the most favorable. When binding free energy decomposition and hydrogen and hydrophobic

statistics were taken into account, **158** was elected the best hit compound. Furthermore, this compound was stable during the simulation. Overall, it has been suggested that the compounds which interact towards the cation binding residue in the central cavity of NorM will have better results, and that good inhibitors should have interaction with a few key residues, Gln-34, Val-35, Gly-38, Phe-63, Tyr-67, Ile-68, Glu-261, Tyr-294, and Asp-377, which will lead to maximum stability and affinity with the NorM transporter [182].

6.1.2. Synthetic Compounds

These compounds have been previously described as MFS inhibitors, namely of the NorA pump. However, similar activity has been described for the MepA pump, of the MATE family. Compound **80** (**Figure 5**), incorporating a bisaryl urea moiety at the 7-position, was able to successfully inhibit approximately 84% of ethidium bromide efflux in a MepA overexpressing strain of *S. aureus* [104]. Phenylquinoline derivatives also proved efficient against the same strain. Differently from what was observed with NorA, the compounds that inhibited MepA most efficiently were **86** (**Figure 5**) and **160** (**Figure 19**), which were able to reduce ethidium bromide's MIC by 16-fold at concentrations of 0.78 and 1.56 μ g/ml, respectively, in the MepA overexpressing strain. Ethidium bromide was chosen, instead of ciprofloxacin, as it is a better MepA substrate and other derivatives were able to inhibit both NorA and MepA efflux pumps, being able to completely restore the activity of ciprofloxacin and ethidium bromide against strains overexpressing such pumps [107]. Paroxetine derivatives (**104**), effective against the NorA pump, also demonstrated potency in inhibiting the MepA pump in *S. aureus*. Oppositely to NorA, paroxetine derivatives do not need the fluorophenyl ring at position 4 to be MepA EPIs. In fact, 4-unsubstituted analogues proved to be more potent than 4-F-phenyl derivatives in inhibiting the MepA pump, as is the case of compound **161** and **162** [122].

Efflux pumps are one of the main causes for antimicrobial resistance. Their inhibition can lead to the restoration of bacteria susceptibility and antibiotic efficacy. Throughout this review, the data presented leads to the conclusion that EPIs are promising compounds and can help reversing the problem of antimicrobial resistance. However, the research into these kind of compounds is still in a very preliminary state, since no EPI is yet under clinical trials.

Even though no general structural features can be pointed out for a molecule to be an EPI, it can be observed that small molecules with high lipophilicity are most likely to fulfill the requirements to inhibit efflux pumps. Hydrophilic compounds can also be transported through porins present in the membrane. Heterocyclic compounds also show potential as EPIs, mainly nitrogen heterocycles, but also fluorine and boron heteroatoms, since the likelihood of bacterial exposure to these atoms is very low. The basis for the MDR pumps inhibition has to do with the penetration rules, and whether

or not a compound is capable of penetrating the bacterial membrane. Therefore, a library of compounds that are able to do that would be something that would bring advances to this study field.

One of the main aims of this review is to present SAR studies, so that pharmaceutical chemists can refine molecular structures, leading to the achievement of more potent and specific compounds. The use of synthetic approaches, either to modify molecules obtained from natural sources, or drugs that are already in therapeutic use, or even to develop novel compounds, is vital for these SAR studies. The search of EPIs derived from natural products comes from the fact that plants need to protect themselves from pathogenic microorganisms, and produce metabolites that act as antimicrobial and EPIs, that synergize with each other. However, the referred compounds are not specific for the pump they have been studied, showing a multitarget character: flavonoids, coumarins, cinnamic acids, among others, present a multiplicity of activities already described, suggesting their promiscuity.

The advantage of assays with existing drugs lies in their safety profile and, most times, their clinical use for combination therapy has been characterized. In some cases, the compounds were even investigated as inhibitors of human efflux pumps, for cancer treatment. This can be regarded as an advantage, leading to a new class of drugs with dual human/bacterial EPI, particularly useful in cancer patients with infections, potentiating the effects of both the anticancer and the antibacterial drugs.

The future in studies in bacterial EPIs should lie either in the search for selectivity for the bacterial target, or in the disclosure of the common features of the universal bacterial EPI, that can successfully target all efflux pumps. The SAR studies gathered herein with the 3D structural information available are expected to accelerate the structure-based design of new EPIs, founded on the existing models.

LIST OF ABBREVIATIONS

3D-QSAR: Three-dimensional quantitative structure activity relationship; ABC: ATP-binding cassette; ADME: Absorption, distribution, metabolism and excretion; ATP: Adenosine triphosphate; BCRP: Breast cancer resistance protein; COX-2: Cyclooxygenase-2; CYP450: Cytochrome P450; EPI: Efflux pump inhibitor; IC₅₀: Half maximal inhibitory concentration; MATE: Multidrug and toxic compound extrusion; MDR: Multidrug resistance; MIC: Minimum inhibitory concentration; MFS: Major facilitator superfamily; MRP: Multidrug resistance protein; MRSA: Methicillin-resistant *Staphylococcus aureus*; NDGA: Nordihydroguaretic acid; NMP: 1-(1-Naphthylmethyl)-piperazine; P-gp: P-Glycoprotein; PAβN: Phenyl-arginine β-naphthylamide; PDB: Protein Data Bank; PSSRI: Phenylpiperidine selective serotonin reuptake inhibitors; RND: Resistance-nodulation-division; SAR: Structure activity relationship; SMR: Small multidrug resistance.

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Title page

Medicinal Chemistry Updates on Bacterial Efflux Pump Modulators

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Medicinal Chemistry Updates on Bacterial Efflux Pump Modulators

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Abstract: Antibiotic resistance is one of the most pressing health issues of our days. It can arise due to a multiplicity of factors, such as target modification, decrease in the drug uptake, changes in the metabolic pathways and activation of efflux pumps. The overexpression of efflux pumps is responsible for the extrusion of drugs, making antibiotic therapy fail, as the quantity of intracellular antibiotic is not enough to provide the desired therapeutic effect.

Efflux pumps can be included in five families according to their composition, nature of substrates, energy source, and number of transmembrane spanning regions. The ABC superfamily are mainly found in Gram-positive bacteria, use ATP as an energy source, and only a limited number of ABC pumps confer multidrug resistance (MDR).

On the other hand, the MFS family, most present in Gram-positive bacteria, and the RND family, characteristic of Gram-negative bacteria, are the most associated with antibiotic resistance. A wide variety of inhibitors have been disclosed for both families, from either natural or synthetic sources, or even drugs that are currently in therapy for other diseases.

The other two families are the SMR, which are the smallest drug efflux proteins known, and the MATE family, whose pumps can also resort to the sodium gradient as an energy source.

In this review, it is intended to present a comprehensive review of the classes of efflux pump inhibitors from the various sources, highlighting their structure-activity relationships, which can be useful for medicinal chemists in the pursuit of novel efflux pump inhibitors.

Keywords: Antimicrobial resistance; efflux pump inhibitors; natural products; synthetic compounds; existing drugs; Structure-activity relationship.

1. INTRODUCTION

Bacteria were discovered the late 19th century as infectious agents. It was, however, half a century later that antibiotics completely revolutionized medicine and became a turning point in human history. Unfortunately, the misruled use of these drugs led to the appearance of resistant strains, and to the concern of a return to the pre-antibiotic era, where the simplest, most treatable infections can become deadly [1-2]. Antibiotic resistance can arise due to multiple causes. The most common are the overuse and/or inappropriate prescribing, as well as extensive veterinary and agricultural use. These factors are especially alarming if it is taken into account the very few new antibiotics that were discovered in the past years [3]. Due to their genetic plasticity, bacteria can develop defense mechanisms in response to environmental threats. As far as antibiotics are concerned, bacteria use two major strategies to adapt to these drugs, which are mutations in genes and the acquisition of resistance genes through horizontal gene transfer. The first strategy can be manifested through modification of the target, decrease in the drug uptake, activation of efflux pumps to extrude the drug or

changes in metabolic pathways. The latter is related to the acquisition of external genetic material through transformation, transduction, or conjugation [4-5]. Recently, vesicle-mediated resistance has also been regarded as a mechanism of resistance acquisition [6].

Efflux pumps are ubiquitous in bacteria, and can be encoded by genes present either in the bacterial chromosomes or in plasmids [7-8]. They concern five families, according to their composition, substrates, energy sources, and number of transmembrane spanning regions: the adenosine triphosphate (ATP)-binding *cassette* (ABC) superfamily, the resistance-nodulation-division family (RND), the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family (**Figure 1**) [7-12].

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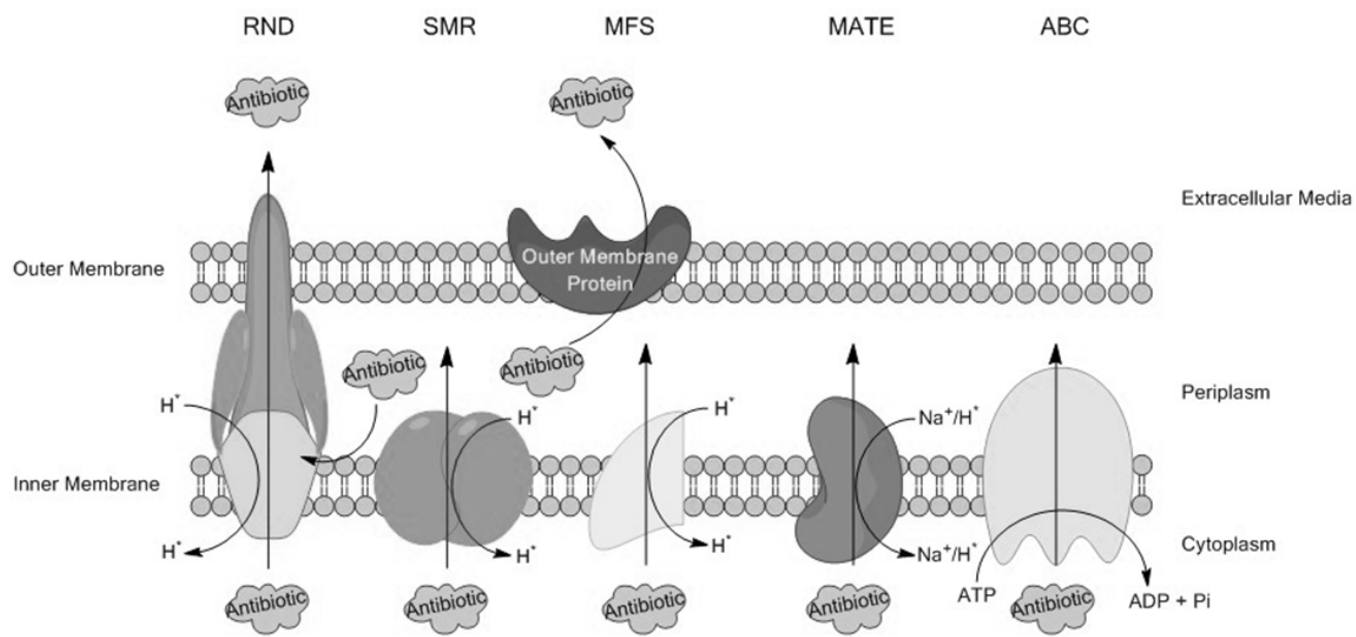


Figure 1 Representation of the five efflux pump families (adapted from [8]).

Efflux pumps can be further divided into two major families, the primary transporters, which hydrolyze ATP as a source of energy, as is the case of the ABC family, and secondary transporters, which use the proton or sodium gradient as a source of energy [13].

Overexpression of multidrug efflux pumps is correlated with an increase in the minimum inhibitory concentration (MIC) of antibiotics, and thus, these pumps can be considered potential antibacterial targets. Therefore, the development of efflux pump inhibitors may be a way of fighting against resistant microorganisms. Studies have been carried out on the structure of efflux pumps, as well as on their mechanisms of binding and extrusion, leading to the possibility of structure-based design of efflux pump inhibitors (EPIs). The rationale for the development of these EPIs could be, for instance, the inhibition of the energy sources required for the ability to extrude antibiotics, and the development of compounds that compete with the antibiotics for their extrusion. However, the main challenge lies in the fact that many compounds that present EPI activity and are already used for the treatment of other diseases are toxic for human cells in the concentrations required to present EPI activity and, therefore, clinically useless [7-9, 13].

Even though there are already several reviews which describe different classes of EPIs [11, 13-31], most focus on a particular source of compounds or on a class of pumps or bacteria. Herein, it is intended to present a comprehensive review of the several classes of EPIs from natural and synthetic sources and from existing drugs in therapeutics and to highlight structure-activity relationship studies in the referred efflux pumps (ABC, RND, MFS, SMR and MATE), which can be useful for medicinal chemists in the pursuit of novel compounds for this goal.

2. ABC

The ABC transporters are primary transporters, and they exist in every membrane systems, whether the organism is prokaryotic or eukaryotic. P-Glycoprotein (P-gp) is a very well known human ABC transporter, involved in the resistance of cytotoxic drugs used in cancer therapy [32-34], whose modulation has been regarded as a study field [35-37]. Structurally, an ABC transporter consists of four conserved domains, two transmembrane domains, and two cytoplasmic domains, which bind to nucleotides and, therefore, are involved in ATP binding [32, 38]. In fact, the nucleotide binding domains are responsible for hydrolyzing ATP, which will lead to conformational changes in the attached transmembrane domains, making it possible for substrates to cross the lipid bilayer of the membrane, either in an inwards or an outwards fashion [39].

The bacterial transporters are highly selective for sugars, amino acids, metallic cations, organo-iron complexes, vitamins, and antibiotics [32, 40]. They are found in Gram-positive bacteria, such as *Enterococcus faecalis* and *Staphylococcus aureus*, and confer resistance to macrolides and bacitracin. However, only a limited number of ABC pumps are associated with MDR, which means that ABC pumps do not play a pivotal role in conferring antibiotic resistance in bacteria [32-33].

As such, bacterial ABC transporter inhibitors are scarce. The few EPIs discovered in this context are mainly against MsrA (**Figure 2**, PDB 1NWA), whose tridimensional structure has been elucidated [41].

The structures of the ABC inhibitors that will be further detailed throughout this section are comprised in **Figure 3**.



Figure 3 Crystal structure of MsrA, from *Mycobacterium tuberculosis* in complex with protein-bound methionine (PDB 1NWA).

2.1. ABC inhibitors

2.1.1. Natural Products

The search for natural products comes from the reason that plants produce antimicrobials to protect themselves from exogenous organisms. It is thought that plants can produce anti-MDR compounds to fight the microbial resistance phenomenon. Carnosic acid (**1**) was one of the major products isolated from the plant *Rosmarinus officinalis*. When tested against the macrolide resistant strain of *S. aureus*, expressing the MsrA pump, compound **1** displayed favorable results, showing an impressive 8-fold potentiation of erythromycin [42].

An extract of *Lycopus europaeus* led to the isolation of two new isopimarane diterpenes, methyl-1 α -acetoxy-7 α ,14 α -dihydroxy-8,15-isopimarandien-18-oate (**2**) and methyl-1 α ,14 α -diacetoxy-7 α -hydroxy-8,15-isopimarandien-18-oate (**3**), which showed no antibacterial activity, but were able to reduce erythromycin's MIC from 256 $\mu\text{g/ml}$ to 128 $\mu\text{g/ml}$, therefore causing a 2-fold potentiation. This behavior was similar to other isopimarane diterpenes isolated in this study, as well as for 5,9-dihydroxygeranylinalool (**4**) and 9-

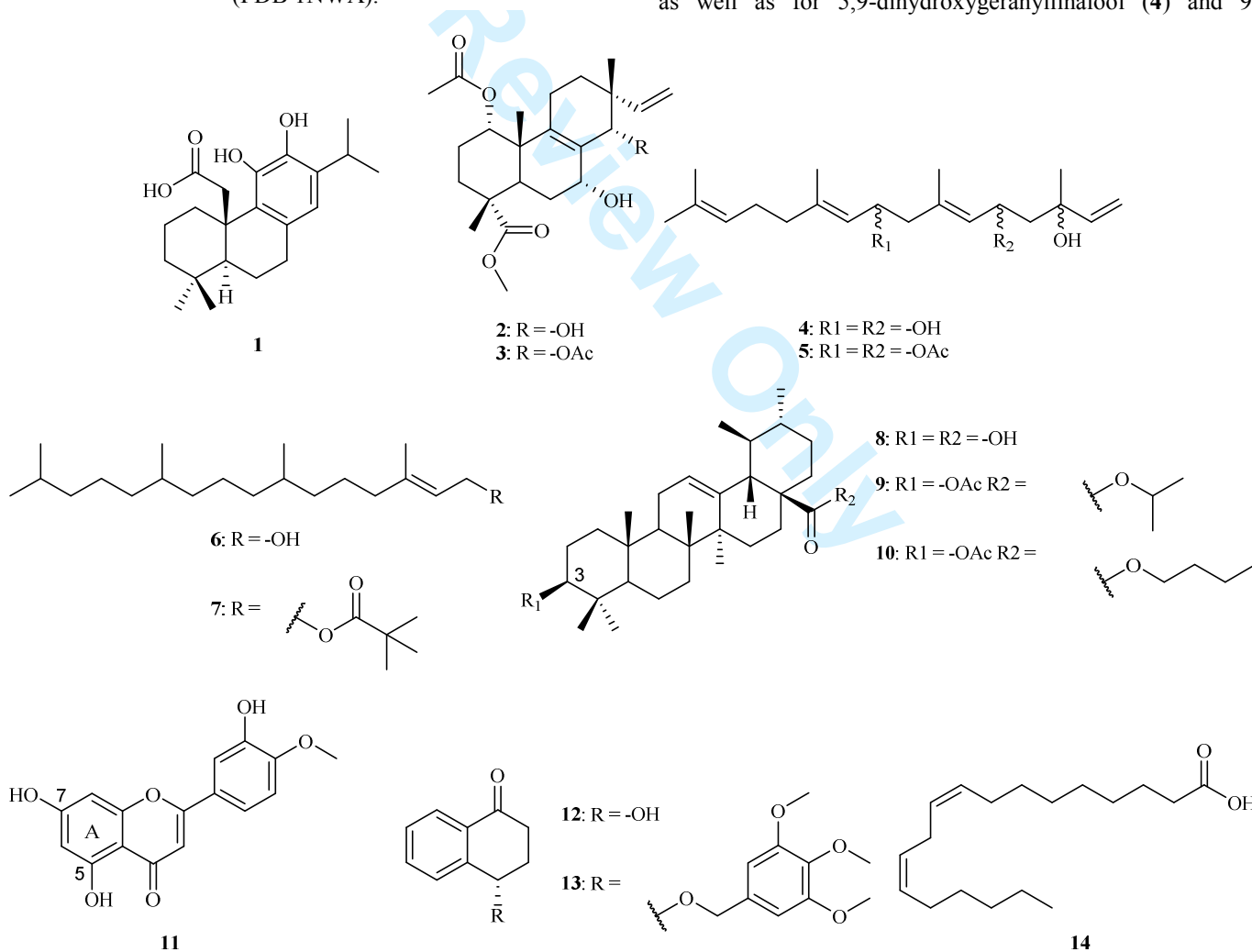


Figure 2 Structures of ABC inhibitors **1** – **20**. Ac = Acetyl.

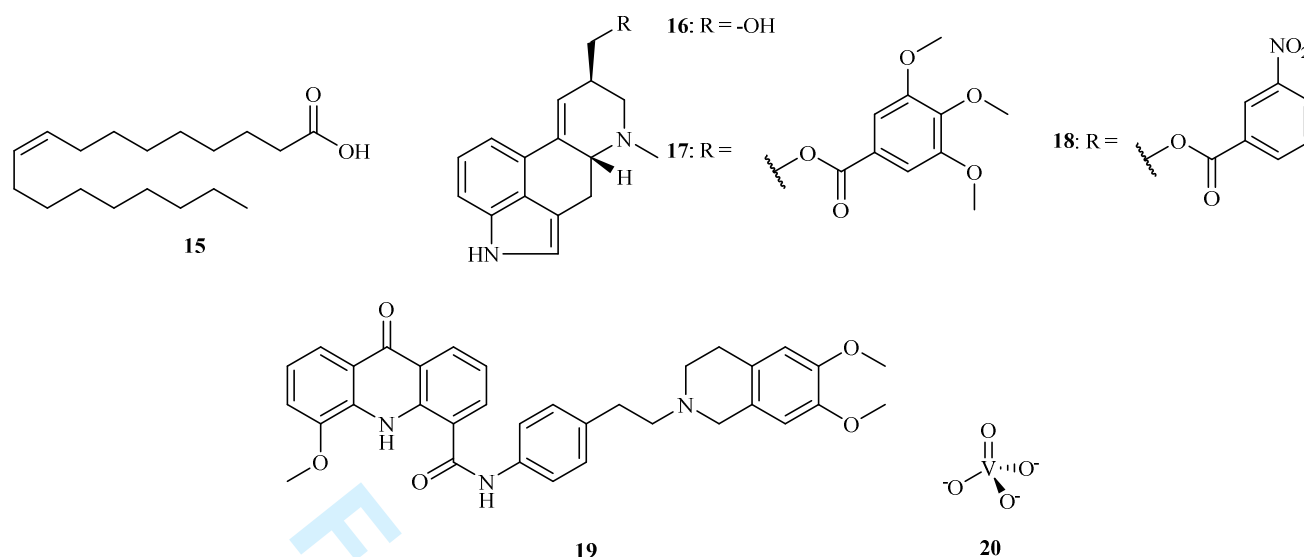


Figure 3 (cont.)

acetoxyl-5-hydroxygeranylinalool (**5**), two diterpenoids [43-44].

Phytol (**6**) is an acyclic isoprenoid, and its EPI activity against the ABC pumps of *E. coli* was assessed in combination with nalidixic acid. Additionally, 15 derivatives were synthesized. One derivative in particular, compound **7**, was able to decrease the MIC of nalidixic acid by 16-fold, and four other derivatives reduced the MIC by 8-fold. This effect was caused by the inhibition of ATP-dependent efflux pump and down-regulation of the efflux gene. *In silico* absorption, distribution, metabolism, and excretion (ADME) analysis was performed, taking into account parameters such as solubility in water, human intestinal absorption, plasma protein binding, blood-brain barrier penetration, hepatotoxicity and cytochrome P450 (CYP450) inhibition. Results showed all five active derivatives presented low permeability into the central nervous system and displayed moderate intestinal absorption. In terms of plasma protein binding level, all five compounds exhibited levels below 90%, suggesting favorable distribution of the compounds into the blood. They also did not present hepatotoxicity, unlike reserpine, making them good lead compounds [45].

Ursolic acid (**8**) is a pentacyclic triterpenoid isolated from diverse natural sources. Its wide activity includes anti-inflammatory, antimicrobial, diuretic, and antitumor activity, among others. When tested against *Escherichia coli* strains that were resistant and susceptible to nalidixic acid, ursolic acid did not display intrinsic antibacterial activity. However, ursolic acid (**8**) was able to reduce the MIC of nalidixic acid by 2-fold, when both drugs were used in combination. This synergy led to further research on this scaffold. The first derivative prepared was the 3-*O*-acetyl ursolic acid, and showed a 4-fold decrease in the MIC of nalidixic acid. The lipophilic nature of this derivative, allied with the increased potency, led to the synthesis of other lipophilic ester derivatives. The methyl and ethyl esters of 3-*O*-acetyl ursolic acid did not show any increment in the activity. However, a

further increase in the ester carbon chain increased the drug reversal potential by 2-fold, in comparison to 3-*O*-acetyl ursolic acid, against the resistant strain. This is the case of the isopropyl (**9**) and the *n*-butyl (**10**) derivatives. The addition of a pentyl group led to a decrease in activity. Furthermore, ursolic acid (**8**), and derivatives **9** and **10**, reduced the MIC of tetracycline by 2-fold, 8-fold, and 8-fold, respectively. When these compounds were tested against ATP-dependent efflux pumps, it was found that derivatives **9** and **10** were successful in inhibiting these pumps in terms of the liberated inorganic phosphate. Molecular modeling studies with the YojI pump, using maltose/maltodextrin transport ATP-binding protein as a homology model, showed that **9** was bound to the nucleophilic Ser-78, to the aromatic Phe-81, Trp-85 and Leu-86, the acidic Glu-94 and Glu-144 and the basic Ala-79, Ala-139, Ala-140, Ala-141, Ala-143, Arg-74, Arg-122, Arg-146, Leu-107, Val-80, Lys-96, and Ile-123 in the nucleotide binding domain. Derivative **10** was also subjected to the same kind of studies using the same model, and was predicted to bind in the same region [46].

A study with flavones and isoflavones from the plant *Sophora moorcroftiana* revealed that diosmetin (**11**) was able to inhibit three drug resistant strains of *S. aureus*, including the ABC family MsrA overexpressing strain, potentiating the effects of norfloxacin and streptomycin [47]. These results corroborate the works of Chan *et al* (2013), who had previously studied the effects of diosmetin (**11**) as an EPI [48]. This group observed a synergistic effect of this flavonoid with erythromycin, which was 5-fold greater than that of verapamil [48].

4-Hydroxy- α -tetralone (**12**) isolated from the plant *Ammannia multiflora*, showed enhancement against nalidixic acid resistant strains of *Escherichia coli*, prompting the synthesis of derivatives in order to infer their mechanism of action. Five derivatives were synthesized, and it was shown that compound **13** was able to inhibit the expression of the ABC transporter protein gene *yojI*, which encodes the ABC

protein YojI, and was successful in combination with tetracycline [49]. Molecular modelling studies with compound **13** and a homology model of the YojI protein revealed the following bound residues: Ser-78 (nucleophilic), Leu-86, Ile-123, Ala-137, Ala-140, Ala-141, and Ala-143 (hydrophobic), Glu-144 (acidic), Arg-122 (basic), Phe-81 (aromatic), Glu-144 (acidic), Arg-146 and Lys-96 (basic), and Trp-85 (hydrophobic), the same region as noted for ursolic acid derivatives **9** and **10**. Compound **13** showed good affinity and strong hydrophobic interaction with the YojI protein, corroborating the fact that this compound acts by the inhibition of ATP dependent efflux pumps [49].

The plant *Portulaca oleraceae* is used in folk medicine for its anti-inflammatory, anti-septic and diuretic effects, among others. A study of its fatty acids demonstrated that linoleic (**14**) and oleic acids (**15**) had synergistic activity with macrolides for the MsrA overexpressing strain of methicillin-resistant *S. aureus* (MRSA). In fact, erythromycin's activity was enhanced when in combination with either of these two compounds, likely through the inhibition of the MsrA ATP dependent pump. Since these fatty acids are essential for humans, low toxicity is expected, making them promising agents to use in combination with antibiotics [50].

Lysergol (**16**) is a clavine alkaloid of the ergoline family. Recently, its EPI activity was studied, and derivatives were synthesized [51]. The compounds were tested against susceptible and resistant to nalidixic acid strains of *E. coli* and a tetracycline resistant strain. While not possessing antibacterial activity, lysergol (**16**) showed synergy with nalidixic acid at the concentration of 10 µg/ml, reducing its MIC by 8-fold in the resistant strain of *E. coli* and 4-fold in the susceptible strain. Of the thirteen semi-synthetic derivatives prepared, derivatives **17** and **18** reduced nalidixic acid's MIC by 8-fold, twice as much as lysergol (**16**), in the susceptible strain. A 4-fold potentiation of nalidixic acid was observed for the three compounds when tested against the resistant strain. As for the tetracycline resistant strain, all three compounds were able to reduce tetracycline's MIC by 8-fold. Further studies were performed, in order to conclude whether or not these compounds were capable of interfering with ATP-dependent efflux pumps, and it was proven that all three compounds inhibited ATPase activity, concerning the liberation of inorganic phosphate. A down-regulation of the gene *yojI* was also observed when the tetracycline resistant strain was treated with lysergol (**16**) and its two most potent derivatives, **17** and **18**, which further confirms the activity of these compounds as ABC transporter inhibitors [51].

2.1.2. Existing Drugs

As part of a screening program for P-gp inhibitors, GG918 (**19**) was discovered. This synthetic compound had already showed success in increasing the intracellular concentration of paclitaxel in cancer cells. In *S. aureus*, this compound was able to slightly potentiate the activity of norfloxacin in a strain overexpressing the ABC efflux pump

MsrA, suggesting that it may possess minimal activity over this pump [52].

The human P-gp inhibitors, vanadates, have been studied as inhibitors of the MutS pumps, belonging to the ABC ATPase superfamily. In fact, these compounds have demonstrated activity against the MutS in *P. aeruginosa* and *E. coli*. Decavanadate was the most effective, followed by orthovanadate (**20**), and they act non-competitively, possibly by replacing the inorganic phosphate. The fact that the ABC ATPases inhibited by orthovanadate are phosphorylated leads to the assumption that the low inhibitory effect could be attributed to the absence of a stable phosphorylation state of MutS [53].

3. MFS

The MFS is the most extensively studied family of transporters, present in both Gram-positive and Gram-negative bacteria, and comprises uniporters, symporters and antiporters. It also constitutes the largest family of secondary transporters, with over 10 000 sequenced members. Its substrates are diverse, and can range from ions, to carbohydrates, lipids, amino acids, nucleosides, among others. As many members of the MFS originate bacterial MDR, these transporters can pose as a good system for modulation studies [7, 19, 38, 54].

With the elucidation of the crystal structure of the MFS pump EmrD of *E. coli* (Figure 4, PDB 2GFP) [55], a general structure could be proposed for MFS transporters: twelve transmembrane helices that form a compact structure, with four of these transmembrane helices facing away from the interior cavity and the rest forming the internal cavity, constituted mostly by hydrophobic residues [7].

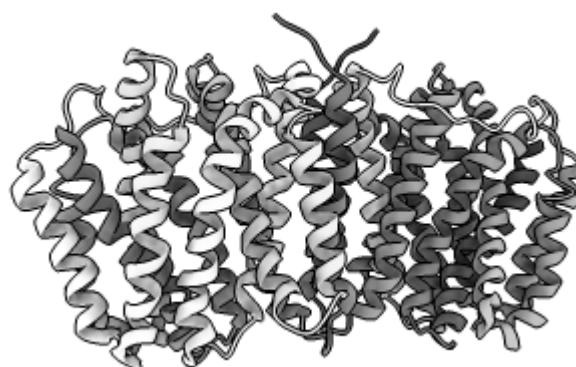


Figure 4 Crystal structure of EmrD, from *E. coli* (PDB 2GFP).

The efflux of drugs is only possible if the carrier alternates between two conformations, so that the substrate can bind to the convenient site in either side of the membrane. However, a limitation concerning the energy involved to displace the exposed hydrophilic surface of a protein can be inferred. It has been proved that either the oligomerization domain or the transport subunit provide the support needed to the movement of the substrate binding site, through a rigid-body

rotation of the substrate binding domain, referred to as the “elevator mechanism” [56-57]. For this family, the most extensively studied efflux pump is the NorA pump of *S. aureus*.

The inhibitors that have proven activity against efflux pumps of the MFS family are gathered in **Figure 5**.

3.1. MFS inhibitors

3.1.1. Natural Products and Derivatives

Terpenes and terpenoids, existent in the essential oils of plants, have showed promising therapeutic potential in various fields. Therefore, studies about EPI potential of these

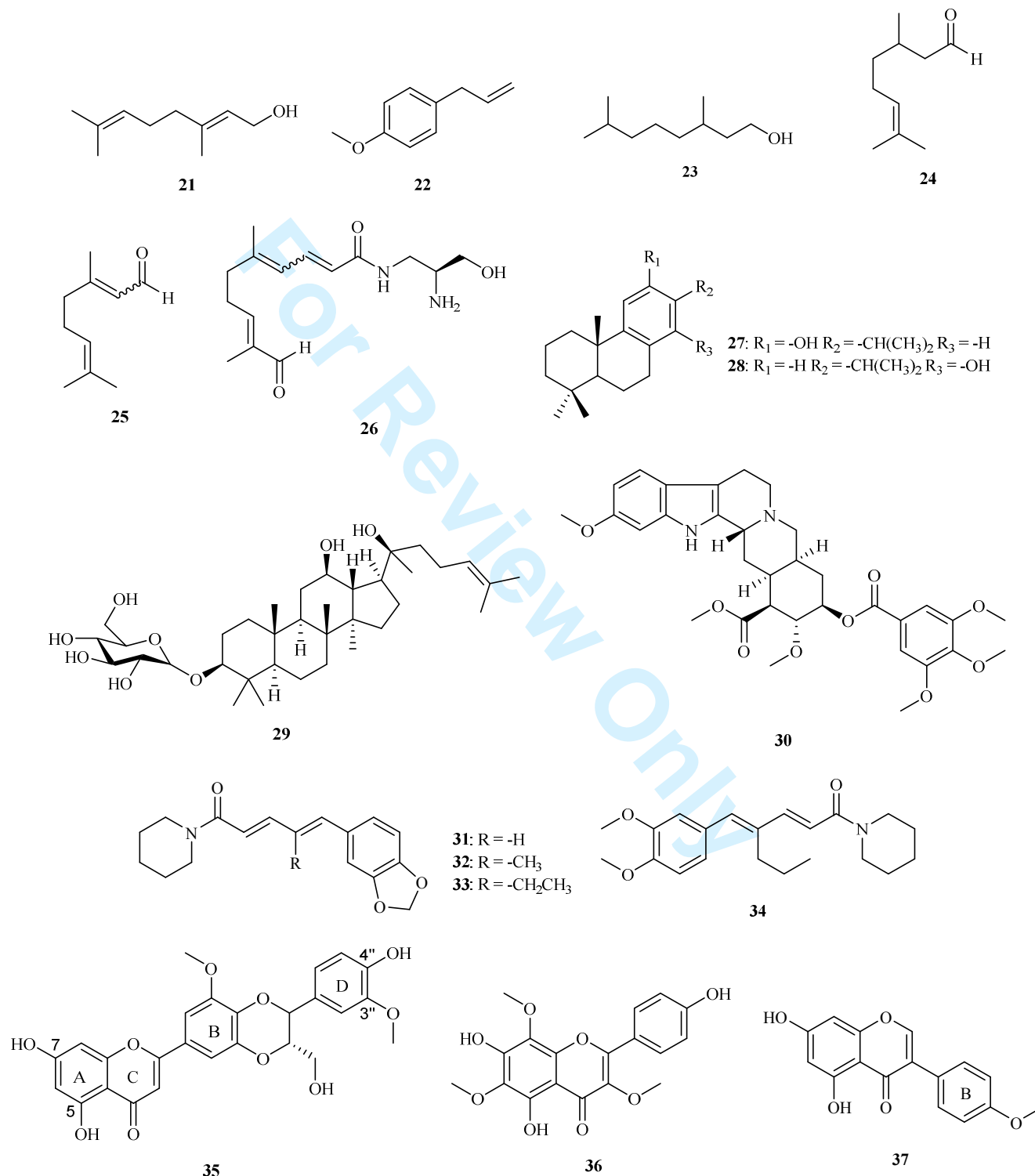


Figure 5 Structures of MFS inhibitors 21 – 115. Ac = Acetyl.

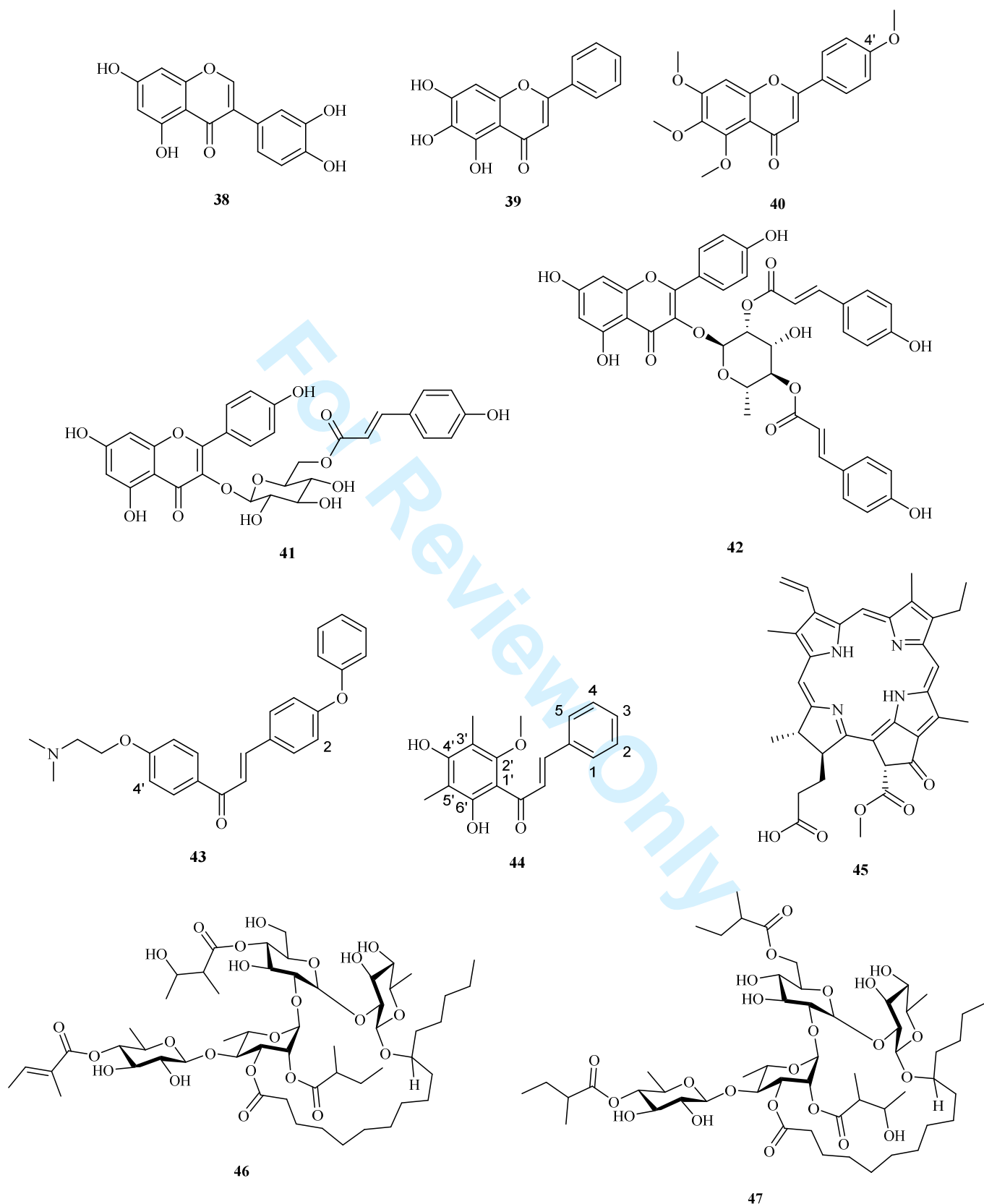
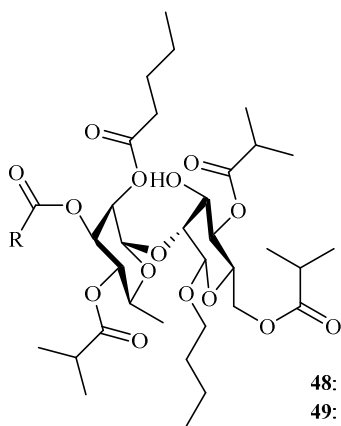
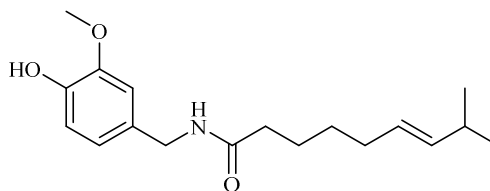


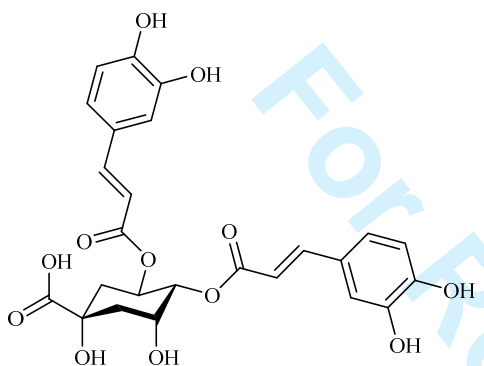
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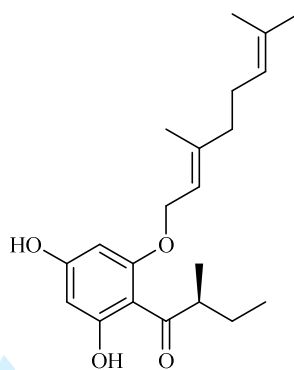
49: R = CH₂CH₂CH₂CH₃



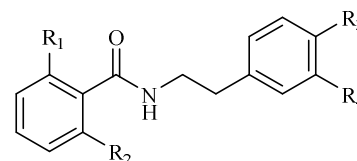
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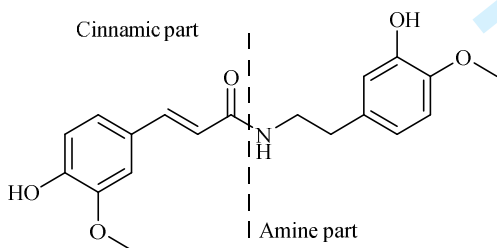
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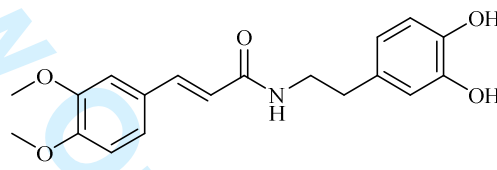
53: R₁ = R₂ = R₃ = R₄ = -H

54: R₁ = R₂ = -OH R₃ = R₄ = -H

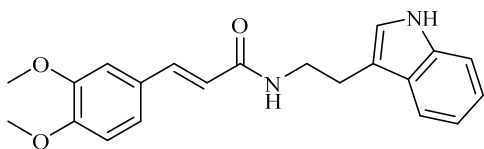
55: R₁ = R₂ = -H R₃ = R₄ = -OCH₃



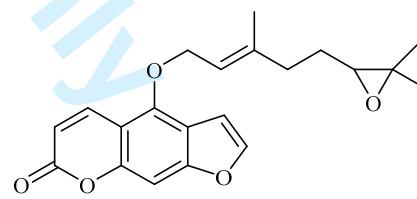
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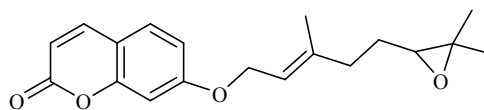
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Figure 5 (cont.)

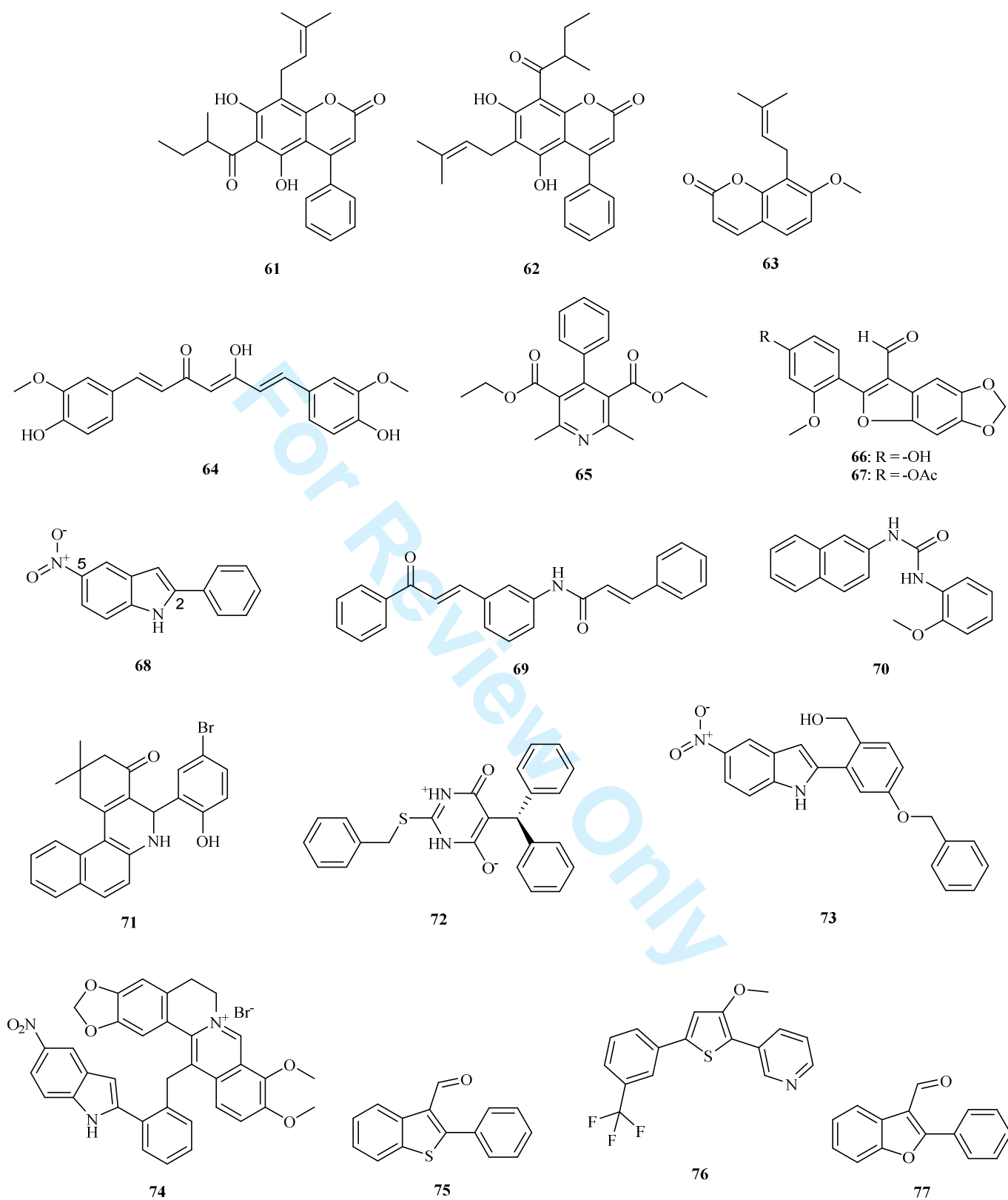


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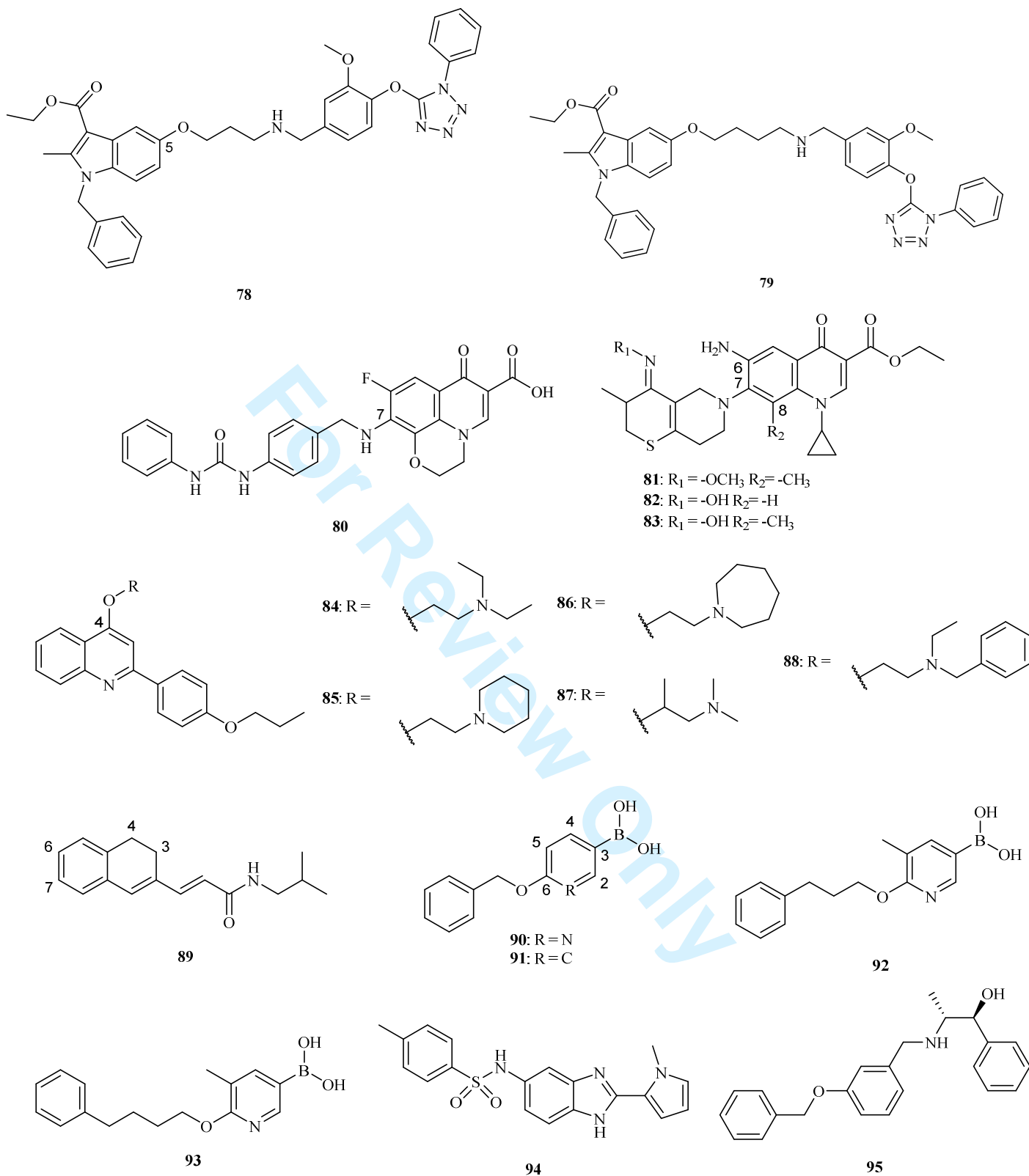


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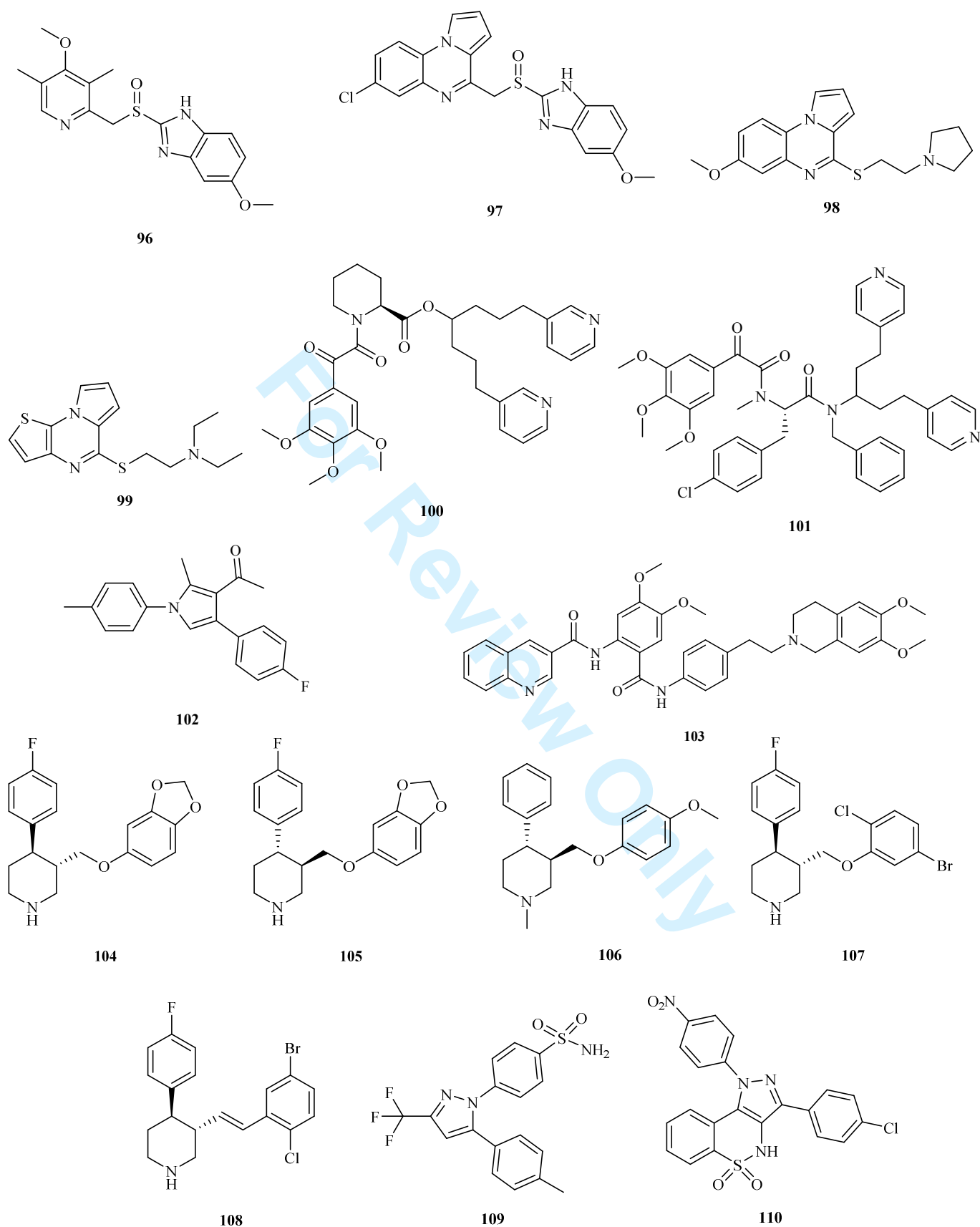


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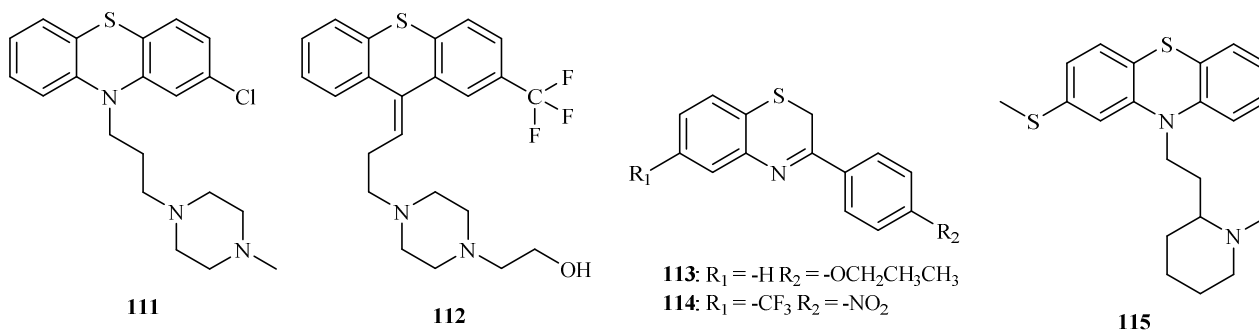


Figure 5 (cont.)

compounds were carried out. Monoterpenes nerol (**21**), estragole (**22**), and dimethyl-octanol (**23**) were tested against *S. aureus*. Compounds **21** and **23** were able to enhance the activity of norfloxacin, decreasing its MIC against the NorA overexpressing strain while not displaying intrinsic antibacterial activity. These open chain terpenes proved to be more effective than **22**, which carried a benzene ring, at inhibiting the NorA pumps [58].

Citronellal (**24**) and citral (**25**) are monoterpenes, and were chosen as starting material for the synthesis of new amide derivatives. The basis for this study was the fact that aromatic amides are recognized as potent EPIS, whereas alkyl, alkenyl, and alkylnil amides had not yet been explored. The compounds obtained were 5,9-dimethyldeca-2,4,8-trienoic acid amides and 5,9-dimethyldeca-2,8-dienoic acid amides, whose activity for the potentiation of ciprofloxacin in *S. aureus* was assessed. Of the first 22 synthesized compounds, seven were able to reduce ciprofloxacin's MIC by 4-fold. Further modifications were performed, and the C-9 methyl group was replaced by an amide, yielding compounds with similar potency as the previous ones. Derivatives subjected to hydrogenation led to decreased activity, which reveals that unsaturation is an important feature for antimicrobial potentiation. The mechanism of inhibition was assessed through ethidium bromide efflux, confirming these compounds act by inhibiting NorA, being compound **26**, a 9-formyl-5-methyldeca-2,4,8-trienoic acid amide, the most potent inhibitor [59].

Ferruginol (**27**), a diterpene, showed efficiency in inhibiting the efflux of ethidium bromide, proving its activity as a NorA inhibitor. It also showed a decrease in norfloxacin efflux in *S. aureus* [60]. Totarol (**28**), a phenolic diterpene, also showed a reduction in ethidium bromide efflux, suggesting activity against NorA pump, even though its mechanism is not yet known [61].

The triterpene ginsenoside 20(*S*)-Rh2 (**29**), a trace compound from red ginseng, also enhanced the effect of ciprofloxacin against *S. aureus*, through a NorA-mediated efflux inhibition, promoting the antibiotic accumulation in the bacteria [62]. Reserpine (**30**) is a natural alkaloid derived from *Rauwolfia vomitoria*, known as an anti-hypertensive

agent, with inhibitory activity against human P-gp and breast cancer resistance protein (BCRP) [63-64]. Reserpine (**30**) has proven to be an effective inhibitor of the Bmr multidrug transporter in *Bacillus subtilis*, diminishing the efflux of ethidium bromide. Its structural analogue rescinnamine also had EPI activity, in a smaller extension than reserpine (**30**) [65]. Through DNA sequencing of the *bmr* genes of mutants, it was shown that reserpine (**30**) interacts with the residues Phe-143, Val-286, and Phe-306 of Bmr pumps, inhibiting drug transport [19]. Compound **30** has shown potential as an EPI for the inhibition of NorA efflux pumps in *S. aureus*, which are structurally related to Bmr pumps, reversing susceptibility to fluoroquinolones, demonstrating reductions up to 4-fold in MIC for some tested strains. It has been shown that **30**'s effect is more pronounced in hydrophilic fluoroquinolones, such as ciprofloxacin [66]. The mechanism by which **30** performs the inhibitory activity is through direct binding to the transporters that mediate drug efflux, acting as a competitive inhibitor [19, 67]. Reserpine (**30**) also succeeded in inhibiting the chloramphenicol efflux by CmlR1 and CmlR2 of *Streptomyces coelicolor*, the ciprofloxacin and norfloxacin efflux in *Listeria monocytogenes*'s pump Lde, and the kanamycin and fusidic acid efflux by LmrS from a MRSA clinical isolate. On the other hand, for *L. monocytogenes*, **30** was not able to reverse susceptibility to linezolid and lincomycin, which suggests that **30** does not completely overlap with the structure binding sites of efflux pumps, being the inhibition dependent on the type and nature of the substrate [19]. However, reserpine (**30**) shows neurotoxicity at the concentration required to inhibit NorA, leading to the need to investigate new inhibitors [67]. Piperine (**31**), an alkaloid found in the fruits of *Piper nigrum*, was found to be an inhibitor of ciprofloxacin efflux in *S. aureus*, reducing the MIC by 2-fold. It is thought that compound **31** acts as a direct inhibitor of drug efflux. Compound **31** inhibits drug transport of the pump MdeA, potentiating the effects of benzalkonium chloride, doxorubicin, daunorubicin, novobiocin, tetraphenylphosphonium chloride, rhodamine 6G, virginiamycin and mupirocin [17, 19-20]; it also inhibits ciprofloxacin efflux by NorA [68].

Given these positive results obtained with piperine (**31**), further research into this scaffold was performed. Kumar *et al.* (2008) performed a screening of about 200 structurally diverse compounds for the inhibition of NorA efflux pump, choosing ethidium bromide as a substrate, since the only mechanism through which this compound is expelled is active transport (68). About 50 compounds were able to increase the intracellular concentration of ethidium bromide by 4-fold, while not displaying antibacterial activity. Five of these compounds were more potent than piperine (**31**), being three of these compounds selected for further studies: **32**, **33**, and **34**, being **32** and **34** more potent than **31** by 40-fold [18, 68]. Assumptions regarding structure-activity relationship (SAR) studies of these molecules could be drawn (**Figure 6**).

specific microbial MDR inhibitor, as flavonoids with P-gp inhibitor properties usually possess alkylated 7-O groups. In fact, when added to a wild type strain of *S. aureus*, compound **35** caused a decrease in norfloxacin's MIC to 0.25 µg/ml. Furthermore, when tested with berberine and palmatine, **35** appeared to completely inhibit NorA, suggesting it acts as a non-competitive inhibitor [71].

A number of hydnocarpin-type flavolignanes were synthesized in order to perform SAR studies. Flavolignanes with and without phenolic groups at the 5 and 7 positions had comparable activity. Diosmetin (**11**), the parent compound, presents hydroxyl groups in the A ring and was the most potent compound. Peracetate derivatives of **35** have approximately the same potency as their parent compounds,

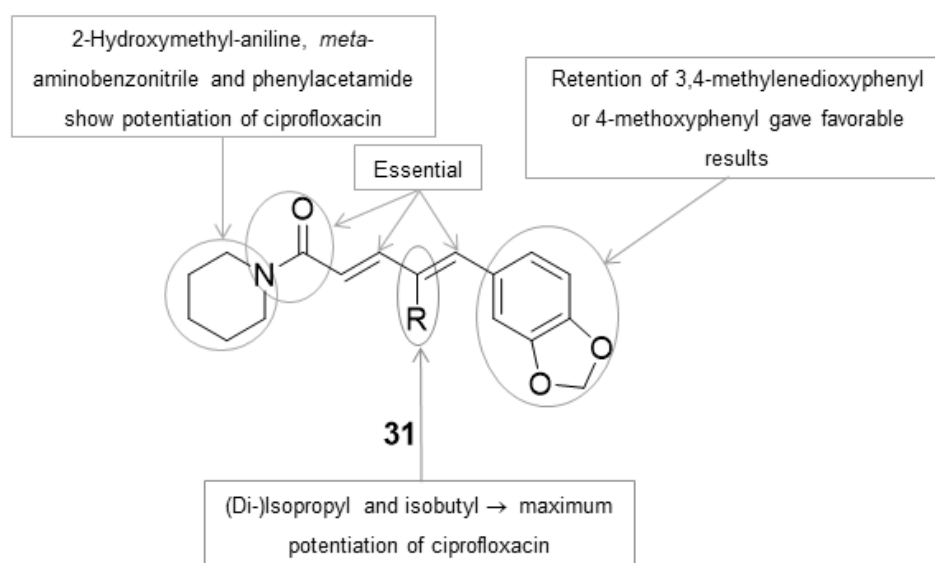


Figure 6 Conclusions from SAR studies for piperine and its derivatives.

It was demonstrated that the introduction of an alkyl group at C-4 position, mainly an isopropyl, isobutyl or di-isopropyl group, coupled with the substitution of the piperidinyl moiety with an aromatic amine, such as aniline, would lead to maximum potentiation of ciprofloxacin; if the aniliny moiety is substituted with a 2-hydroxymethyl group or a nitrile, regardless of the positional isomery, ciprofloxacin activity would increase. Replacing the piperidinyl moiety with a phenylacetamide yields good potentiation of ciprofloxacin, reducing its MIC by 4-fold, regardless of the isomery. Unsaturation is crucial for the potentiation, as well as the amide. Lastly, the retention of 3,4-methylenedioxyphenyl or 4-methoxyphenyl groups in the 1,3-benzodiol moiety also gives favorable results [68-70]. Plants of the *Berberis* species have the ability to produce the alkaloid berberine, which is a plant secondary metabolite produced in response to microbial invasion, whose activity is enhanced by a flavolignane also produced by these species, 5'-methoxyhydnocarpin (**35**) [71]. Compound **35** exists in a mixture of anion and neutral compound at physiological pH, since it has a phenolic group with a pKa of 7.3. Due to the acidic properties of this 7-OH group, **35** is likely to be a

and could be considered prodrugs, if deacetylated by *S. aureus*, which seems unlikely. Considering the D ring, compounds bearing a 3''-methoxy-4''-hydroxy moiety displayed good activity, and 3'',5''-dimethoxy-4''-hydroxy moieties conferred a slightly less active compound. As for the B ring, the presence of an H or an OH is not critical for activity [72]. In addition, a flavone isolated from *Alkanna orientalis*, sarothrin (**36**), blocked ethidium bromide efflux by *S. aureus*, suggesting its potential activity as a NorA inhibitor [73].

Biochanin A (**37**), an isoflavone, also showed potentiation of norfloxacin and berberine against wild-type *S. aureus* and *Bacillus megaterium*. An analogue of luteolin, orobol (**38**), was quite active as a NorA inhibitor, unlike luteolin itself. It was shown that flavones which have a single methoxy in its B-ring are more potent than flavones with two methoxy groups in the B-ring [74].

Another flavone, baicalein (**39**), was capable of potentiating the effect of ciprofloxacin in *S. aureus*. Studies aimed at measuring the efflux of ciprofloxacin and pefloxacin, a poor NorA substrate, were performed, and showed that compound **39** was much more potent at restoring ciprofloxacin

sensitivity than pefloxacin, leading to the conclusion that this flavone acts as a NorA efflux inhibitor, even though its mechanism remains unknown [75].

Maia *et al.* (2011) identified six flavonoids from the plant *Praxelis clematidea*: apigenine, genkwanin, 7,4'-dimethylapigenin, trimethylapigenin, cirsmartin, and tetramethylscutellarein (**40**) [76]. These methoxylated flavones showed no antibacterial activity, but were able to reduce the MIC of norfloxacin at least by 2-fold and up to 16-fold, as was the case of **40**, at a concentration of 8 µg/ml, being therefore considered NorA efflux pump modulators. It is thought that the lipophilicity of the flavonoids, strengthened by the presence of methoxyl groups, is responsible for this activity [21]. Presence of a methoxy in the 4' position proved to be decisive for activity, as flavonoids containing this group were more potent. The number of methoxy groups is also essential for potency, being the most methoxylated compounds the most active [76]. Tiliroside (**41**), an amphipathic kaempferol glycoside, reduced the concentration of antibiotic needed to inhibit the growth of bacteria. The lipophilicity of the flavone moiety may be a determinant factor for the activity displayed. Tiliroside (**41**) showed no antibacterial activity against *S. aureus*, but showed a decrease in the MIC of fluoroquinolones up to 16-fold, when at a concentration of 64 µg/ml. The reduction in the MIC of biocides, such as ethidium bromide and benzalkonium chloride, was impressive, with the MIC of acriflavine showing a decrease by 128-fold at the concentration of 64 µg/ml and 32 µg/ml. These results suggest that **41** might additionally act on pumps other than NorA [77].

Kaempferol 3-*O*- α -L-(2, 4-bis-*E-p*-coumaroyl)rhamnoside (**42**) was also able to potently inhibit in a concentration dependent manner NorA-mediated ethidium bromide efflux in *S. aureus*. This compound also increased the antibiotic effect of norfloxacin against a wild-type and a NorA overexpressing strain of *S. aureus*. However, it has to be considered that **41** and **42** are flavonoid glycosides and cinnamoyl esters, and that their activity can become compromised with the activity small intestine β -glucosidases or plasma esterases, even though the activity might possibly lie in the kaempferol moiety, with **41** and **42** acting as prodrugs. The authors hypothesized that if the activity of compound **42** is compromised, this compound could be used as adjuvant of ciprofloxacin for topical infections [78].

Since **42** showed promising results as a NorA EPI, the same group performed a screening of a library of 117 chalcones, due to their structural similarities with the coumaroyl substituents [79]. Ten out of the 117 screened chalcones presented medium efflux inhibition at 20 µg/ml. The most potent chalcones, such as **43**, bear a dimethylaminoethoxy moiety, which might disrupt the proton gradient, thus eliminating the energy source of NorA. Five of these chalcones possessed a hydroxyl group at the 4'-position, and potentiated the activity of berberine against *S. aureus* and *B. cereus*, suggesting that this substituent is also important for activity. The compound with a methoxy group at position C-2 has shown to present the best activity. The majority of

chalcones tested showed a 4-fold reduction of ciprofloxacin's MIC in *S. aureus*, suggesting the presence of a NorA efflux pump inhibitory activity. Compound **43** showed 8-fold and 16-fold reduction of the MIC of ciprofloxacin at 6.25 and 12.5 µg/ml, respectively. However, it also showed antibacterial activity at 25 µg/ml. Some chalcones also presented antiparasitic activity, namely against *Leishmania major* and *Plasmodium yoelii* [79].

Another chalcone, 4',6'-dihydroxy-3',5'-dimethyl-2'-methoxychalcone (**44**), showed good activity as a MDR, causing complete growth inhibition at a very low concentration (3.3 µg/ml) when combined with a sub inhibitory concentration of berberine. When tested in combination with berberine, erythromycin, and tetracycline against strains of *S. aureus*, there was an increase in the activity of all tested compounds in the wild-type *S. aureus*, indicating a mode of action likely related to the NorA pump. This compound was also tested against *B. cereus*, potentiating the effects of the tested antibiotics as well, and displaying a favorable effect, particularly in the case of berberine, where a 30-fold increase in the activity was observed [80].

Pheophorbide A (**45**) is an intermediate of the natural breakdown of chlorophyll. Concentrations as low as 0.5 µg/ml of pheophorbide A with sub-inhibitory concentrations of berberine were enough to completely inhibit *S. aureus* growth. When extended to fluoroquinolones, this compound also proved efficient in potentiating the effect of norfloxacin by 4-fold in a wild-type strain, being its action directed towards NorA [81].

Orizabins, oligosaccharides from resin glycosides, were evaluated for their efflux pump inhibitory activity on strains of *S. aureus*. These amphipathic compounds showed synergy with norfloxacin, whilst showing no antibacterial activity by themselves. Orizabin IX (**46**) completely inhibited the growth of a NorA overexpressing strain at 1 µg/ml, while orizabin XIX (**47**) reversed norfloxacin resistance by 4-fold at 25 µg/ml for the same strain [82]. Murucoidins, which are also resin glycosides, strongly potentiated the action of norfloxacin against a *S. aureus* NorA overexpressing strain by 4-fold at concentrations ranging from 5 to 25 µg/ml. Stoloniferin I potentiated the same activity by 8-fold at a concentration of 5 µg/ml [83].

A study aiming to find new EPIs from the plant *Geranium caespitosum* led to the isolation of novel acylated neohesperidosides. It was found that two compounds, compound **48** and **49**, were successful at restoring susceptibility to berberine, norfloxacin, and ciprofloxacin at sub inhibitory concentrations, possibly due to NorA inhibition [84].

Capsaicin (**50**), the major constituent of the fruits of the *Capsicum* genus, is a known P-gp inhibitor. Studies were performed, in order to evaluate if its inhibitory activity was also applied to MDR pumps in bacteria. In a strain of *S. aureus* overexpressing the NorA efflux pump, and therefore resistant to fluoroquinolones, **50** proved efficient in increasing the susceptibility to ciprofloxacin, while reducing the emergence of ciprofloxacin-resistant mutants. Molecular

modelling studies of the complex capsaicin (**50**)-NorA, using the glycerol-3-phosphate transporter to predict the structure of NorA, showed that compound **50** aliphatic chain extended into NorA's hydrophobic cleft. Furthermore, a weak hydrogen bond between Arg-98 and the aryl hydroxyl contributed to the stability of the complex [85].

Studies in the aqueous extract of *Artemisia absinthium* showed promise in the inhibition of MDR efflux pumps. It was proven that this activity came from caffeoylquinic acids. At a concentration of 20 μM , 4'-5'-*O*-dicaffeoylquinic acid (**51**) potentiated the activity of berberine by 16-fold and of norfloxacin, by 10-fold against a wild-type strain of *S. aureus*, while not displaying effects in a *norA*-deleted strain, confirming this compound's NorA inhibition ability [86].

Olympicin A (**52**) is an acylphloroglucinol, found in the aerial parts of *Hypericum olympicum*. This molecule had some efflux impeding ability, proven by the improvement of intracellular accumulation of enoxacin in a NorA overexpressing strain of *S. aureus*, at 50 $\mu\text{g/ml}$ [87].

Riparins are natural alkylamides found in the plant *Aniba riparia*. Since the extraction of these compounds comes with very low yields, synthetic approaches were developed. As such, the fundamental core of riparins, Rip-A (**53**), was synthesized and derivatives were prepared, Rip-B (**54**) to E (**55**). Rip-E (**55**) showed good growth inhibitory activity against *S. aureus*, attributed to their lipophilic nature and the presence of hydroxyl groups at the benzamide moiety.

Extracts of *Mirabilis jalapa* have shown good results in reversing fluoroquinolone resistance in strains of *S. aureus* overexpressing the NorA efflux pump. One compound in particular, *N-trans*-feruloyl-4'-*O*-methyldopamine (**56**), caused an 8-fold reduction in the MIC of norfloxacin, making it a potential lead in the search for structurally similar synthetic alkylamines [89].

Synthesis of derivatives was undertaken, taking *N-trans*-feruloyl-4'-*O*-methyldopamine (**56**) as a model. The aim was to identify active compounds from natural sources and, as such, couplings between cinnamic acid derivatives and natural-occurring amines were chosen. SAR criteria was taken into account, as it is known that substitution of the aromatic rings, methoxy or hydroxyl substitution, double bonds and aromatic ring nature in the amine part influence the activity. The lead compound was *N-trans*-3,4-*O*-dimethylcaffeoyl dopamine (**57**) since it showed the same activity as the natural compound. It was found that, for the cinnamic moiety, a hydroxyl substitution on the aromatic ring appears to be better than a methoxy group or unsubstituted derivatives and the double bond is essential for activity. As for the amine part, trisubstitution on the aromatic ring increases antibacterial activity, at the cost of decreased efflux pump inhibition. Methoxy substitution yields compounds with better results than hydroxyl substitution, which was better than no substitution and tryptamine combinations showed the best results (Figure 7). Among the

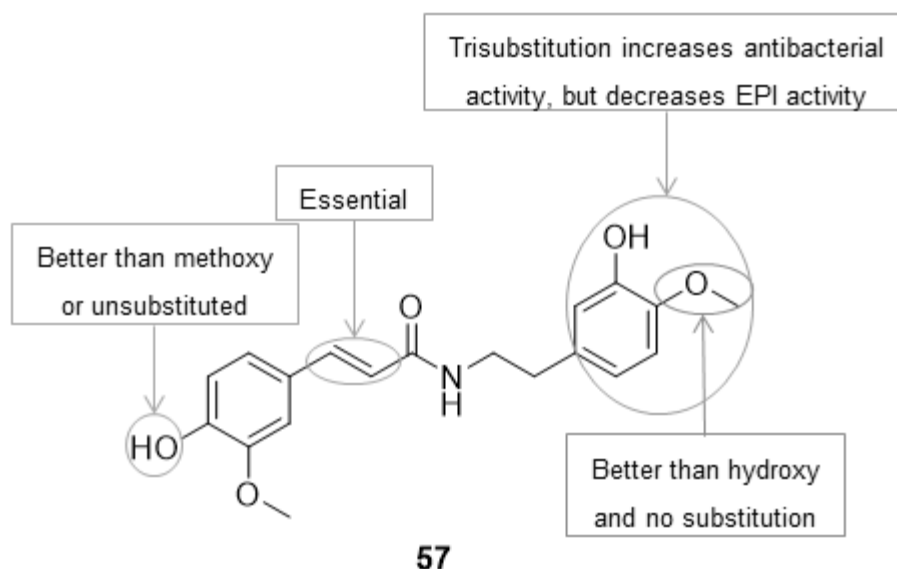


Figure 7 Conclusions from SAR studies for compound **57**.

Oppositely, Rip-B (**54**), with two methoxy groups at the phenyl-ethyl moiety did not present antibacterial activity, presenting instead a decrease in the MIC of ciprofloxacin and norfloxacin, similarly to reserpine. Combined with the ability of decreasing ethidium bromide's MIC, the activity of compound **54** was attributed to NorA inhibition, suggesting its potential as an EPI [88].

compounds tested, compound **58** showed potentiation of norfloxacin comparable to that of reserpine (**30**). At a concentration of 30 μM , **58** showed good results for the inhibition of ethidium bromide efflux. For these compounds, it was shown that the activity reaches its highpoint when the phenyl ring is substituted with two hydroxyls [89].

Grapefruit oil fractionation led to the isolation of three compounds, two coumarin derivatives and one bergamottin

derivative. The bergamottin epoxide (**59**) showed a reduction of ethidium bromide's MIC by 6-fold on different MRSA strains, and a 20-fold reduction in norfloxacin's MIC. This reduction was also achieved when the coumarin epoxide (**60**) was present, and suggests an activity towards NorA efflux pumps [90].

Coumarins isolated from *Mesua ferrea* were also studied for their efflux pump inhibitor activity against clinical strains and NorA overexpressing strains of *S. aureus*. Seven coumarins were tested, and showed good results in inhibiting ethidium bromide efflux. Two compounds, **61** and **62**, showed potential EPI activity against the NorA overexpressing strain and a MRSA strain, using norfloxacin as a substrate, which makes them desirable compounds for the synthesis of derivatives [91].

Screening of natural products with P-gp inhibitory effects (92, 93) led to the identification of two compounds with NorA inhibitory activity, osthol (**63**) and curcumin (**64**) [36, 92-93]. Curcumin (**64**), derived from the rhizomes of *Curcuma longa*, has additionally already shown activity as an anti-inflammatory, antioxidant, antiviral, and anticancer agent [36, 92-93]. These compounds were effective in reducing the MIC of ciprofloxacin by 4- and 8-fold, respectively [94].

A penta-substituted pyridine, 2,6-dimethyl-4-phenylpyridine-3,5-dicarboxylic acid diethyl ester (**65**), was isolated from the rhizomes of *Jatropha elliptica*. This compound, when in association with ciprofloxacin, showed a strong effect against NorA efflux pumps in *S. aureus* [95].

A methanol extract of the plant *Dalea spinosa* yielded the isolation of six compounds, whose EPI potential was analyzed against wild-type *S. aureus* and mutant strains. It was shown that spinosan A (**66**), at a concentration of 48 µg/ml, and its acetate **67**, at a concentration 42 µg/ml, were able to decrease berberine's MIC by 8- and 62-fold, respectively, against the wild-type. Other compounds were also able to reduce berberine's MIC, but to a lesser extent. Compound **67** also managed to decrease berberine's MIC in the NorA overexpressing strain, suggesting inhibitory activity in this efflux pump [96].

3.1.2. Synthetic Compounds

The INF series (compounds **68-72**) resulted from an initial screening of 9600 structurally diverse compounds, in order to find NorA inhibitors for *B. subtilis*, not expressing Bmr. The NorA substrate chosen was ethidium bromide [67].

Since this screening did not focus on a specific chemical group, the most active compounds were divided into several groups. Several indole derivatives were active, which did not come as a surprise, since reserpine (**30**) itself has an indole moiety. Another large group was the trichloromethylamine containing compounds, which were not further explored as their likelihood to be toxic was high. Biphenyl urea derivatives were also active, and other compounds, with no obvious similarities, showed activity as well. Five compounds were selected for further tests, INF 55 (**68**), INF 240 (**69**), INF 271 (**70**), INF 277 (**71**) and INF 392 (**72**).

These compounds were active at concentrations of 5 µg/ml or less, and included the most potent compound, **72**, the most potent indole, **68**, and the most potent biphenyl urea, **71**. Derivative **72** was able to reduce resistance to ethidium bromide and ciprofloxacin by 8-fold at a concentration of 0.4 µg/ml. All five inhibitors were more potent than reserpine (**30**) at promoting the activity of ciprofloxacin in *S. aureus* and, by being structurally different, it was hypothesized that a potent, nontoxic lead would be identified. These compounds also proved effective in inhibiting the Bmr multidrug transporter in *B. subtilis* and two compounds, **68** and **71**, also enhance fluoroquinolone activity in *Streptococcus pneumoniae* [67].

The results obtained with INF 55 (**68**) in *S. aureus* as an inhibitor of the NorA efflux pump led to an increased interest in this kind of scaffold. SAR studies regarding the C-5 proved that substitution in this carbon is crucial for activity. Substitution with a nitrile group leads to retention of potency, and carbonyl based electron-withdrawing groups at C-5 would result in molecules with no activity [97]. Three dimensional-quantitative structure-activity relationship (3D-QSAR) studies predicted that 2-aryl indole derivatives would be NorA inhibitors. Functionalized INF 55 (**68**) showed a decreased MIC for berberine, a MDR transporter substrate. Berberine is an amphipathic cation alkaloid. From the series of 2-aryl-5-nitroindoles synthesized by Samosorn *et al.* (2009), the alcohol **73** was the most effective in inhibiting berberine efflux, as well as other antibiotics, such as ciprofloxacin, in *S. aureus* [98]. Later, the same group developed a strategy that consisted in a molecule presenting dual action: an antibiotic and a MDR inhibitor. Therefore, they combined berberine, a hydrophobic cation that is a MDR substrate, with INF 55 (**68**), a MFS inhibitor. These efforts yielded compound **74** [17-18, 99]. In terms of antibacterial activity in *S. aureus*, the hybrid was about 100 times more active than berberine alone, and the difference was even higher when tested against a NorA overexpressing strain of *S. aureus*. *Enterococcus faecalis* also presented resistance to berberine, but was susceptible to **74**. [99] Synthesis of sulfur derivatives was achieved based on the premise that the activity of **68** is less dependent on the nitro group than the 2-arylindole. Benzothiophene, thiophene, and benzofuran derivatives, such as compounds **75**, **76**, and **77**, respectively, were able to synergize with ciprofloxacin and ethidium bromide, increasing their intracellular concentration and leading to the conclusion that these compounds inhibit NorA in *S. aureus*. [100]

Taking into account the fact that the indole NH is not essential for efflux pump inhibition, Samosorn *et al.* (2009) simplified the indole moiety of berberine-based hybrids, along with the effect of a methylene ether linking group. Their results showed that a hybrid molecule with oxygen in the linkage chain had stronger antibacterial and MDR pump inhibitory activity than the original **74** [101]. On the other hand, removal of the indole moiety produced compounds with lower dual activity, and this structural simplification led to compounds with low antibacterial activity, but with retained NorA pump inhibitory activity. These data suggest

that while the indole moiety is not necessary for NorA pump inhibition, it is required for the antibacterial activity of hybrid compounds [101].

A recent study identified a novel class of NorA EPIs with a polysubstituted indole moiety. Twenty molecules were highly active in inhibiting ethidium bromide efflux in *S. aureus*. However, the best results were obtained with compounds **78** and **79**, which were derivatives that presented three and four carbon atom chains, respectively. Compound **78** presented the best half maximal inhibitory concentration (IC₅₀), with a concentration of 1.8 μM. Compound **79** exhibited similar IC₅₀ (2 μM) and ethidium bromide inhibition as **78**. Compounds with shorter or longer carbon chains did not present such good results, leading to the conclusion that three or four carbons make up the right distance between the indole scaffold and the basic center, thus presenting the best inhibitory effect. When tested for their synergy with ciprofloxacin, both **78** and **79** were able to restore the antibiotic's activity [102]. It was found that the substitution of the C-5 in the indole results in a potent EPI,

MepA pump systems from the MATE family [17-18, 104]. Fluoroquinolones containing a thiopyranopyridine moiety at the C-7 position were synthesized and their activity was tested against strains of *S. aureus*. However, these derivatives did not seem to be NorA substrates. Further investigations into thiopyranopyridinylquinolone esters were carried out. Some of the synthesized compounds displayed better inhibitory activity than reserpine (**30**), namely quinolone esters **81**, **82** and **83**, and showed significant MIC reduction of ciprofloxacin. Compounds **82** and **83** also showed a 32-fold MIC reduction of ciprofloxacin for strains of *S. aureus* overexpressing MepA pumps from the MATE family [18, 105]. In terms of established SAR, these compounds definitely do not need the fluorine atom at the C-6 position for the antibacterial activity of thiopyridinylquinolones. A methyl at the C-8 position yields a compound with enhanced activity against Gram-positive bacteria for 6-amino derivatives. The thiopyranopyridine moiety at the C-7 position is crucial for NorA and MepA inhibition (**Figure 8**) [105].

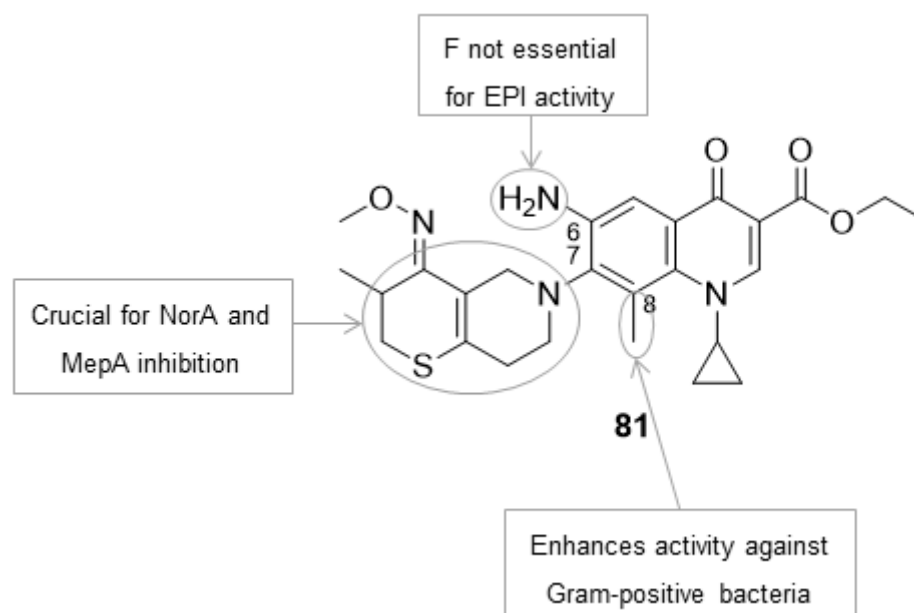


Figure 8 Conclusions from SAR studies for fluoroquinolone **81** derivatives.

particularly if there is a propoxy chain carrying terminal cyclic amino groups, which appears to be crucial for NorA inhibition at low concentrations. The presence of the *N*-benzyl moiety preserves inhibition and contributes to the modulation of the biological effects and ADME properties, depending on its substituents [103].

Another successful example of a hybrid antibacterial/EPI is the fluoroquinolone scaffold accommodating a bis-aryl urea EPI moiety at the C-7 position. This kind of compounds was successful in inhibiting NorA pumps in *S. aureus*. Compound **80**, which incorporates a bis-aryl urea motif into the ofloxacin core, proved to be a potent inhibitor of MFS efflux pump systems, achieving 84 % inhibition rate at 10 μM. Moreover, these compounds also successfully inhibit

Derivatives of 2-(4-propoxyphenyl)quinolone were synthesized based on the premise that its large hydrophobic area and ability to establish an electrostatic interaction would make them suitable inhibitors of NorA. These derivatives also mimic the quinolone antibacterial core and possess a versatile scaffold, making it prone to be very simply chemically modified. These classes of compounds were obtained by performing modifications in the 2-phenyl-4*H*-chromen-4-one moiety, which is a common feature of flavone and flavolignane EPIs [106].

Studies showed that the *O*-substituted 2-phenyl-4-hydroxyquinoline derivatives displayed the best activity as NorA inhibitors. Compounds **84** and **85** showed synergistic activity against a strain of *S. aureus* overexpressing NorA

efflux pumps comparable to reserpine (**30**) and, comparing the results obtained with a mutant strain, absent of NorA, it was found that these compounds are able to completely restore the antibacterial activity of ciprofloxacin in the resistant strain [18, 106]. Preliminary SAR studies for this new class of EPIs showed that the best activity is when the quinolone core is replaced with the 2-phenyl-4-hydroxyquinoline group with an alkylation in the C-4 hydroxyl group. 2-Ethylamino chains inserted at the N-1 position of the quinolone nucleus or at the C-4 hydroxyl of the quinoline moiety provided compounds with better activity than those carrying the same chain at the C-2 of the phenyl ring. The best substituent for the C-2 phenyl ring appears to be the C-4' propoxy group (**Figure 9**) [106].

reduction in this strain for ciprofloxacin's MIC. SAR studies for this emerging class show that the best activities come from the compounds bearing the 2-ethylaminoalkyl chains linked to the C-4 hydroxyl group, such as compound **87**. The inclusion of an aliphatic ring including the side chain nitrogen atom led to retention of activity, whereas inclusion in an aromatic ring led to loss of activity, where the nitrogen nucleophilicity was reduced. The mono-substituted compounds, like **88**, with a benzyl group were well tolerated, and showed higher ethidium bromide inhibitory activity than compounds with two benzyl groups as substituents (**Figure 9**) [107].

3-(Dihydronaphthyl)-propenoic acid amines were evaluated for their EPI activity. Five compounds were synthesized, and

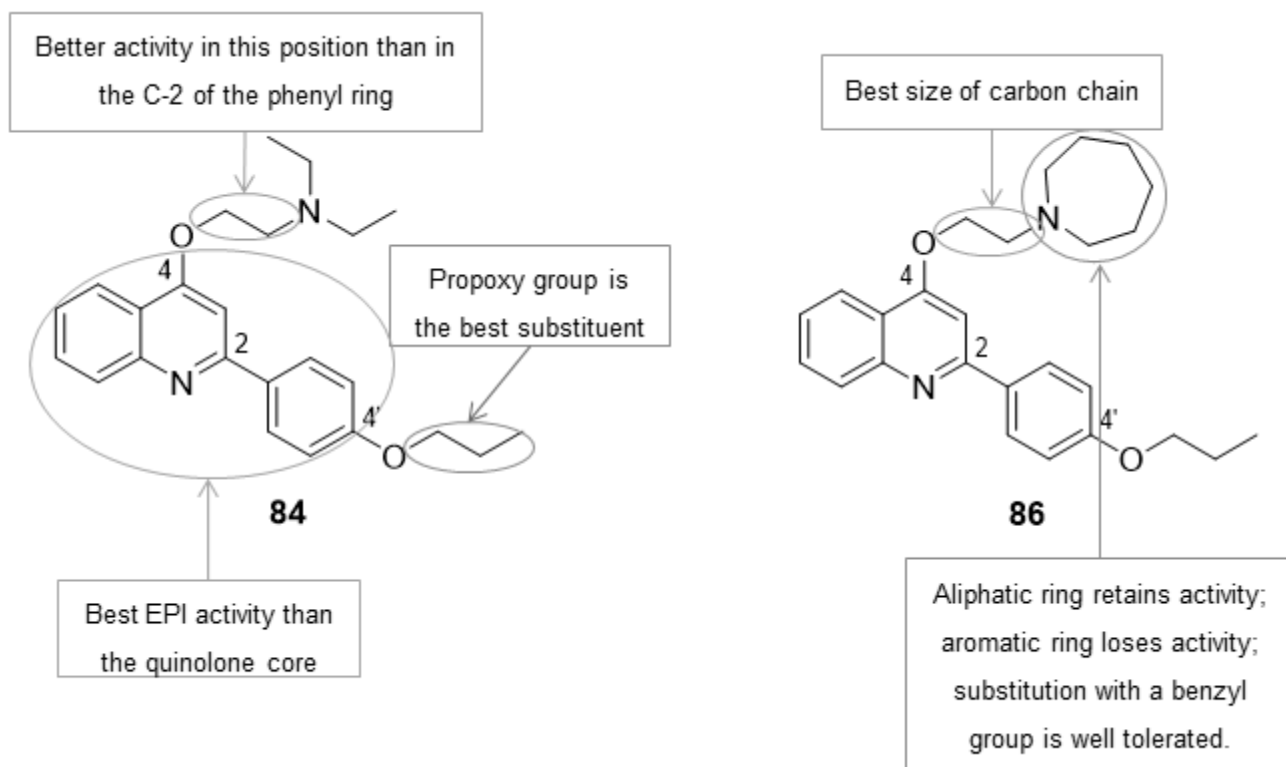


Figure 9 Conclusions from SAR studies for 2-phenyl-4-hydroxyquinoline derivatives **84** and **86**.

Taking these results into account, efforts have been placed to obtain more potent NorA inhibitors, introducing *O*-alkyl or different *O*-alkylamino chains at the C-4 position. The strategy was to determine the essential 3D structural requirements for the inhibition of NorA, based on the NorA EPIs described in literature through the use of computational methods, since the 3D structure of this pump was not elucidated yet. The results led to the building of a possible pharmacophore, which was used to design novel compounds, with different substituents at the C-4 position, which were synthesized and biologically evaluated [107]. The compounds were assayed for their ability to reduce the MIC of ciprofloxacin in *S. aureus* strains, and all compounds displayed this decrease for the strain overexpressing NorA pumps. One compound in particular, **86**, showed a 16-fold

two of them showed good results in inhibiting ciprofloxacin efflux in strains of wild-type and NorA overexpressing *S. aureus*. Further studies were performed, given the low EPI activity of this kind of compounds. Fifteen different compounds were synthesized, taking α -tetralone (**12**) as starting material. Out of these, four compounds showed significant MIC reductions for ciprofloxacin for the NorA overexpressing strain. One compound, **89**, even displayed a 16-fold reduction, while other remained in the range of 4- to 8-fold. These studies allowed SAR to be established. Addition of methoxy or allyloxy substituents at the 6,7-position of the 3,4-dihydronaphthalene leads to lower or no activity, whereas no substitution originated better EPIs. Saturation of the double bonds lowered the potentiating

activity (about 2-fold), while unsaturated derivatives made much more potent compounds [108].

Boronic species have been described for a variety of activities, namely antimicrobial and antineoplastic. Organoboron compounds, oxazaborolidines, boronic esters, and boronic acids are of particular interest, within this class. Fontaine *et al.* (2014) developed studies on these molecules, starting from a screening of a library of approximately 150 compounds, out of which 24 were hit compounds (exemplified with compounds **90** and **91**), and were able to restore the activity of ciprofloxacin by 4-fold at concentrations of 0.5 to 8 µg/ml against the NorA overexpressing strain of *S. aureus* [109]. Preliminary SAR studies demonstrated the need of the boron atom for activity. Most pyridine-3-boronic acids showed best potentiating activity (e.g., compound **90**), whereas pyridine-4-boronic derivatives showed no activity, with exceptions, including a fluorinated ester. The shift of the boronic moiety from C-3 to C-4 led to a loss of activity and the trigonal boronic acid function gave better results than the sodium boronate salts and the potassium trifluoroborate. Concerning the pyridine-3-boronic acids, the most promising compounds appear to be the 6- and the 5,6-disubstituted ones, being the latter compounds less potent. Cyclisation at the C-5 and C-6 positions was well tolerated, as was the introduction of a methyl at the C-5 position. For the benzene analogues, which displayed good activity, cyclisation at the C-3 or C-4 was also well tolerated, as was the introduction of a methyl. A substituent at the *para* position to the boronic moiety proved to be very important. Two compounds were particularly efficient in inhibiting NorA, and did not display intrinsic antibacterial activity and cytotoxicity: the 6-benzyloxy pyridine-3-boronic acid **90**, which displayed the highest activity, potentiating ciprofloxacin by 4-fold at a concentration of 16 µg/ml, and the 4-benzyloxybenzene boronic acid **91** [109].

Compound **90**, being the most promising compound, was considered a hit, and its structure was used as a model for the synthesis of new derivatives. Therefore, the 6-benzyloxy group was substituted with (aryl)alkoxy chains with variable lengths (compounds **92** and **93** as examples), in an attempt to explore the putative hydrophobic binding site of NorA, and substituents were added to the phenyl ring. The ether linkage between the pyridine ring and the 6-benzyloxy group was substituted as well. The first substitution made was the replacement of the 6-benzyloxy group with alkoxy chains of diverse lengths, as to explore the size of the hydrophobic pocket of NorA. The potency of the compounds increased with the increase of the size of the side chain, although the compound with a 11-carbon side chain displayed no activity whatsoever. A new series of compounds with a phenyl ring at the end of the side chain were synthesized, with the aim of enhancing hydrophobicity, and the results showed these compounds were more efficient. It was concluded that the compound with the best performance possessed a side chain with seven carbon atoms and a phenyl group: compounds **92**, 6-(3-phenylpropoxy)pyridine-3-boronic acid, and **93**, 6-(4-phenylbutoxy)pyridine-3-boronic acid, showed the highest

activity in the potentiation of ciprofloxacin (4-fold). The introduction of substituents in the phenyl ring of compound **90** led to unsatisfactory results, with the best compound displaying moderate antibacterial activity. A methyl group in the C-5 position did not show influence on the activity, and the modification of the ether linkage led to varied effects: introduction of a sulfanyl, thus conserving the H-bonding acceptor capacity, led to similar potentiating activity, whereas the introduction of an amine linkage, an H-bond donor, led to a decrease in the activity. Finally, the shift of the benzyloxy group from the C-6 to the C-5 resulted in no activity [110].

Compounds AE-848/42434549 (**94**) and AN-465/42885978 (**95**) were hits in a virtual screening of novel NorA efflux pump inhibitors in *S. aureus*. The structures deemed as active *in silico* were later synthesized and evaluated on their ability to prevent ethidium bromide efflux, as well as ciprofloxacin potentiation. Compound **94** and **95** were as potent as reserpine (**30**) for ethidium bromide efflux and more potent at synergizing with ciprofloxacin. These hit compounds **94** and **95** paved way for the synthesis of new analogues, which allowed SAR studies to be made, in order to assess which parts of their structure were determinant for the inhibition of NorA. For compound **94**, it was shown that limited modifications to the sulfone and amide group do not cause significant changes in activity. On the other hand, modifications to the imidazole ring result in a reduction of activity. Replacement of the 1-methylpyrrole ring with a furan also shows a decrease in the activity of the compound. As for compound **95**, the *O*-benzyl group is essential and the introduction of a benzyl in the secondary nitrogen leads to a complete loss of activity. Furthermore, the methyl group of the 2-(1-phenyl-1-propanol) moiety can be replaced with a 2-(1-phenyl-1-ethanol) portion without a decrease in the activity [111].

3.1.3. Existing Drugs

Several drugs already available for the treatment of various diseases have shown potential to be used as MFS EPIs. Reserpine (**30**), mentioned before, is one of them. Other drugs include verapamil, omeprazole, paroxetine and chlorpromazine. Even though bacterial MFS transporters and mammalian ABC efflux systems, as P-gp, lack structural homology, they share similar substrate profiles, which means that mammalian MDR inhibitors can also present bacterial efflux inhibition. However, the concentrations needed for efflux pump inhibition are too high, which means that toxic effects can arise if these compounds are used for this purpose [18], [112]. In fact, the addition of verapamil to chemotherapy was accompanied with cardiac arrhythmia and hypotension [113]. This leads to the requirement of developing analogues of these existing drugs maintaining the desired EPI activity at concentrations that do not display toxicity [112].

The antiarrhythmic verapamil, a known P-gp inhibitor, has shown moderate NorA inhibitory activity in both *B. subtilis* and *S. aureus*, enhancing fluoroquinolone activity [114-115].

However, the best results were obtained with proton pump inhibitors omeprazole (**96**) and lansoprazole. Therefore, a series of pyrrolo[1,2-*a*]quinoxaline derivatives mimicking the omeprazole structure (**96**) were designed, with the aim of evaluating their EPI activity against NorA. All the eleven derivatives showed a reduction in norfloxacin's MIC, being compound **97** the most active, reducing the MIC by 16-fold at 128 µg/ml. These compounds did not show disturbance of the electrical potential and the transmembrane pH, excluding the alteration of the proton-dependent pump as the mechanism of action. Instead, it is thought that these compounds interact directly with NorA. It was shown that the benzimidazole moiety is critical for the activity of these compounds (**Figure 10**), and replacing it with an imidazole, a pyridine or a pyrrolo[1,2-*a*]quinoxaline ring resulted in loss of potency. Furthermore, if there is a methoxy-substituent in the benzimidazole ring, potency increases. Introduction of chlorine in the C-7 of the pyrroloquinoxaline heterocycle provided an efficient restoration of norfloxacin's bactericidal activity. Finally, the benzimidazolyl group was also important for activity [112].

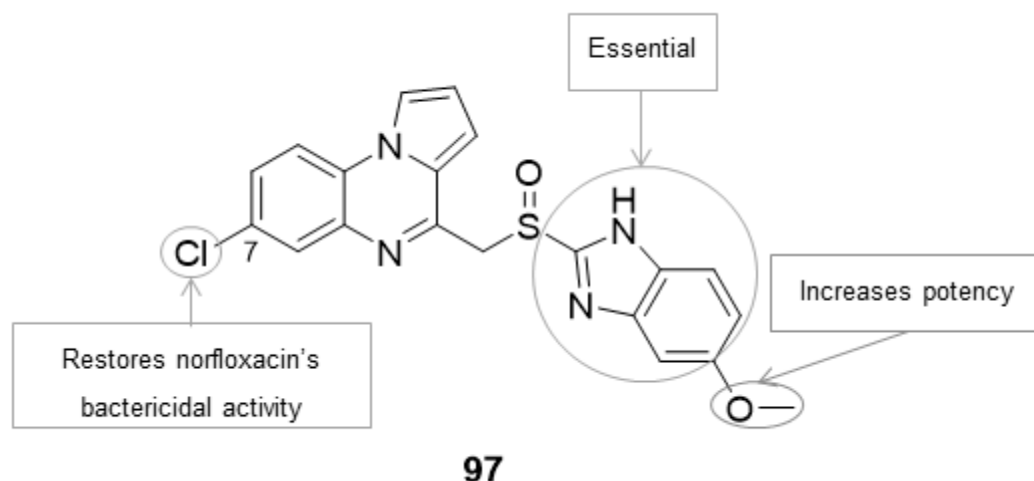


Figure 10 Conclusions from SAR studies for omeprazole derivatives.

This was not the first time that pyrrolo[1,2-*a*]quinoxaline derivatives were described as EPIs. In fact, a previous study by the same group produced 12 new 4-[116]pyrrolo[1,2-*a*]quinoxalines and one pyrrolo[1,2-*a*]thieno[3,2-*e*]pyrazine compound. Two compounds, **98** and **99**, were more active than reserpine (**30**), diminishing norfloxacin's MIC by 16-fold in a NorA overexpressing strain of *S. aureus*. This study showed that 4-[116]pyrrolo[1,2-*a*]quinoxalines and their 7-methoxy analogues were more active, in contrast with the chlorine- and 8-phenyl-substituted compounds, that presented the lowest effects. Replacement of the *N,N*-diethylamino group by pyrrolidine, its isomer with restricted conformation, led to enhanced EPI activity, whereas replacing it with a piperidine ring led to more flexible and, consequently, less active compounds. The pyrrolothienopyrazine **99** moiety yielded the best results,

suggesting that sulfur, an electron-rich atom, potentiates the EPI activity [116].

GG918 (**19**) was discovered in a screening program aimed to identify inhibitors of mammalian P-gp, and was able to increase the concentrations of paclitaxel. This compound did not present antibacterial activity against the strains of *S. aureus* tested. However, the co-administration with norfloxacin resulted in a 4-fold reduction in the MIC of the NorA overexpressing strain [52].

Two mammalian MDR inhibitors, biricodar (**100**) and timcodar (**101**), confer increased drug sensitivity to cells expressing both the P-gp and multidrug resistance protein (MRP)-1 efflux systems. They also show potentiation of multiple antibiotics in Gram-positive bacteria. In fact, both compounds were able to potentiate the activity of ethidium bromide against *S. aureus*, *E. faecalis*, and *S. pneumoniae*. For the specific case of *S. aureus*, both compounds were able to partially reverse fluoroquinolone resistance. The fact that the MIC of ethidium bromide was non-saturable for the three bacteria tested suggests that **100** targets multiple efflux pumps [117].

A series of polysubstituted pyrroles was screened for their P-gp inhibition activity, which made way for the identification of a lead, and the compounds were further investigated for their bacterial efflux pump inhibition activity. A simplified pyrrole alkaloid, **102**, displayed dual activity as both a P-gp and a NorA inhibitor against *S. aureus*. This compound led to 4-fold improvement in the MIC of ethidium bromide in the NorA overexpressing strain. Compound **102** was also able to reduce ciprofloxacin's MIC by 8-fold when at 50 µM [118].

Tariquidar (**103**), a third generation MDR modulator, with activity in inhibiting P-gp and BCRP efflux systems, has shown promise in inhibiting efflux pumps in *S. aureus*. In fact, this compound showed an increase of the isotopic form of ciprofloxacin in all investigated strains, displaying best results in the strain that overexpresses the NorA pump. [119]

The class of phenylpiperidine selective serotonin reuptake inhibitors (PSSRI), which includes the antidepressant paroxetine (**104**), showed efflux-related potential against *S. aureus*, particularly that conferred by NorA. In fact, paroxetine and its isomer, **105**, showed potency with respect to inhibition of this efflux pump against ethidium bromide. The isomer **105** also interfered with the accumulation of norfloxacin by NorA, reducing by 4- to 8-fold its MIC. [120] Femoxetine (**106**) is a paroxetine derivative, with activity as an EPI. [120] Studies were performed as to determine which moieties in paroxetine- and femoxetine-like PSSRI were detrimental for efflux pump inhibition in *S. aureus*, and conclusions from SAR are highlighted in **Figure 11**. Synthesis of derivatives showed that the fluorine atom on the phenylpiperidine moiety is not associated with the activity. The *N*-substitution of the piperidine ring yields different kinds of activity, suggesting that the NH group forms a positive binding contact with the target pump, which does not happen if there is a *N*-substitution (compound **106**). The phenyl ether substituents also play an important role as determining the EPI activity. [121]

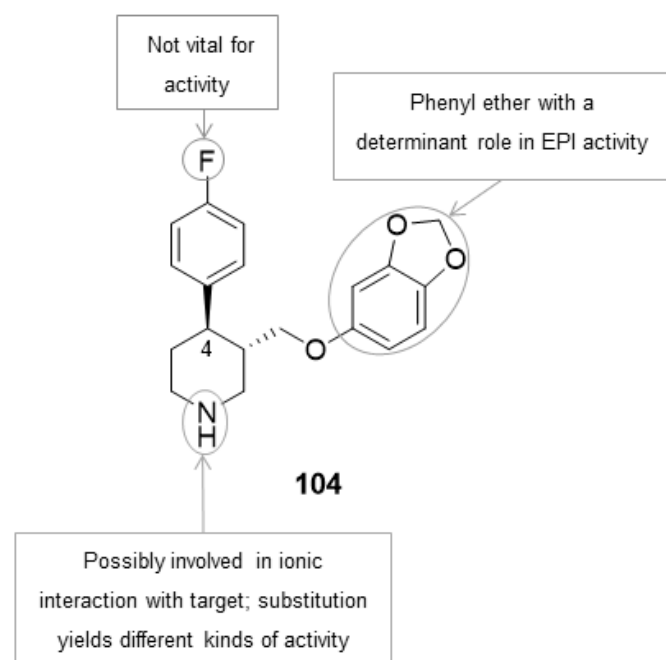


Figure 11 Conclusions from SAR studies for paroxetine derivatives.

Also for this kind of compounds, a study was performed, in order to conclude whether the 4-phenyl ring is required for EPI activity and the effects of changes in the two-atom aryloxymethyl linker, replacing it with a thioether, amine, and alkene linker portions. Compounds **107** and **108** displayed the lowest MIC against *S. aureus*. Synthesis of multiple derivatives proved that the 4-phenyl moiety is not vital for EPI activity and that a variety of two-atom linker groups for the 3-aryl piperidine moiety can maintain EPI activity [122].

The cyclooxygenase-2 (COX-2) inhibitor celecoxib (**109**) has also received attention for its MDR inhibitory activity. There had been a link between COX-2 and *mdr1* gene expression, which has consequences in cancer chemo resistance, and the use of celecoxib (**109**) reversed drug resistance. If this is true for mammalian efflux pumps, for bacteria there is no evidence of a COX-2-like gene, suggesting that celecoxib acts differently in inhibiting MDR in bacteria. A study was carried out in order to prove this hypothesis, which demonstrated that this drug helped in increasing sensitivity to antibiotics in *S. aureus* and *Mycobacterium smegmatis*. These effects probably occur due to the blockage of MDR transporters involved in the efflux of antibiotics, such as ampicillin, ciprofloxacin, kanamycin, and chloramphenicol. The observation of the accumulation of ethidium bromide intracellularly leads to the conclusion that this compound acts as a NorA inhibitor [123].

As celecoxib (**109**) demonstrated such promising activity, analogues were tested, which bore low or none anti-inflammatory activity and possessed the 1,4-dihydropyrazolo[4,3-*c*]-benzothiazine-5,5-dioxide nucleus. Ethidium bromide efflux inhibition was tested first *in silico*, and the 17 molecules that fit the chemical space requirements were then reduced to six, based on the activity prediction. These compounds were then screened for their *in vitro* NorA inhibitory activity, using an overexpressing strain of *S. aureus*. One derivative in particular, compound **110**, showed the best activity concerning ethidium bromide efflux (76.9%) which is better than celecoxib (**109**) (64.5%). When in combination with ciprofloxacin, **110** displayed results comparable to reserpine (**30**) and better than paroxetine (**104**), which makes it a lead compound for the development of inhibitors of this kind [124].

Phenothiazine and thioxanthene derivatives are currently used as neuroleptic and antiemetic agents, and have been described as modest, but broad, antibacterial agents. Although their antibacterial activity is not enough to be clinically relevant, they have shown synergy with standard antibiotic agents, and have also been associated with the inhibition of eukaryotic MDR efflux pumps, such as P-gp. The mechanism through which these compounds exert their antimicrobial potentiation is not yet fully understood. However, it is thought that it might have to do with the inhibition of efflux pumps [125]. A study focusing on the activity of several compounds of these families against *S. aureus* was carried out, with the main aim of showing their potential in inhibiting NorA. The compounds tested, chlorpromazine, fluphenazine, thioridazine, prochlorperazine (**111**), *cis*(*Z*)-flupentixol, and *trans*(*E*)-flupentixol (**112**), displayed intrinsic antimicrobial activity and good results when ethidium bromide efflux was assayed against a NorA overexpressing strains of *S. aureus*. Additionally, phenothiazine **111** and thioxanthene **112** were also able to reduce the proton motor force of *S. aureus*, by reducing the transmembrane potential [125].

Since the phenothiazine moiety proved itself to work as a template for the synthesis of new MDR EPIs, it was chosen to be the scaffold for derivatives with improved activity. The

logic for these studies lied in the elimination of the structural features that were responsible for neuroleptic activity. As such, drastic modifications were made, such as the elimination of one ring of the tricyclic benzothiazine backbone and of the chain linked to the N-10 atom, a tertiary amine whose protonation is crucial for the interaction with the dopaminergic receptor, and the addition of a substituted phenyl ring at the C-3 position, to guarantee better lipophilicity. Therefore, a prototype of new 3-phenyl-2H-1,4-benzothiazines was developed. Preliminary screening on the derivatives using a wild-type *S. aureus* strain, both with and without ciprofloxacin, showed variable intrinsic and synergistic activity. Two compounds, **113** and **114**, displayed the best activity, while displaying no and weak antibacterial activity, respectively. These compounds were able to inhibit NorA efflux of ciprofloxacin, being suggested that **114** was even able to completely inhibit this pump [126].

Thioridazine (**115**), a phenothiazine, was also tested for its antibiotic potentiation, either in racemate or in its enantiomeric forms. All the forms were able to reduce the MIC of oxacillin in *S. aureus* and erythromycin in *Streptococcus pyogenes*, being the L-enantiomer more effective in the last case [127-128].

4. RND

RND pumps are found mostly in Gram-negative bacteria, being the most responsible pumps for MDR in this kind of organisms. They present a wide variety of lipophilic and amphiphilic substrates, which include several classes of antibiotics, antiseptics, dyes, and detergents. RND transporters present a unique tripartite complex, constituted by a minimum of twelve transmembrane segments: the transmembrane pump, the outer membrane channel, and the

periplasmic adaptor protein, which puts the other two components in contact. This structural organization makes the extrusion of substrates directly into the external medium possible [32, 38]. Even though it has always been thought that RND transporters were exclusive to Gram-negative bacteria, it has recently been identified and characterized FarE in *S. aureus*, homolog to the AcrB protein in *E. coli* [129-130].

Within this family, the most studied system is the AcrAB-TolC, from *E. coli*. This efflux pump is composed by three essential parts: a resistance-nodulation-division transporter, AcrB; a membrane fusion protein, AcrA; and a multifunctional outer membrane channel, TolC [7, 131-133]. It transports a wide variety of toxic compounds from the intracellular space directly into the medium, evading the periplasm, and using a proton gradient as the energy source [132-133]. *Pseudomonas aeruginosa* also possesses an efflux system of the RND family, the MexAB-OprM efflux pump, which confers resistance to a wide variety of antibiotics. This was the first and best characterized efflux system in bacteria. It was found that AcrB (**Figure 12**, PDB 4ZLJ) is homolog to MexB (**Figure 12**, PDB 2V50), being their structure very similar [7, 134].

Figure 13 comprises all the RND pump inhibitors that will be discussed in the following section.

4.1. RND inhibitors

4.1.1. Natural Products and Derivatives

One study revealed that the essential oil of *Helichrysum italicum* can reduce the resistance to chloramphenicol in *E. aerogenes*, *P. aeruginosa*, and *A. baumannii*. These data suggested that one or more EPIs are present within the essential oil since it was shown that geraniol (**116**) produced

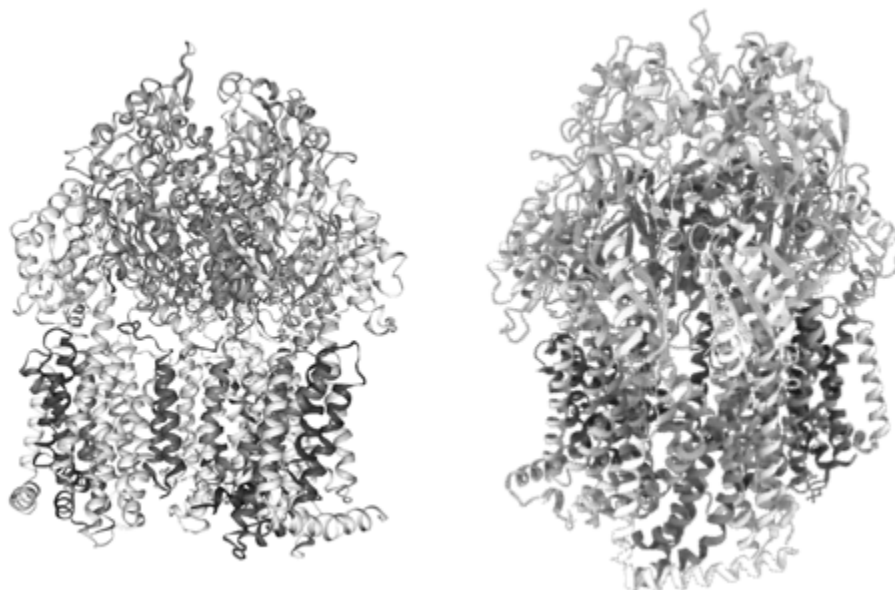


Figure 12 Crystal structures of AcrB (left, PDB 4ZLJ) of *E. coli* and MexB (right, PDB 2V50) of *P. aeruginosa*.

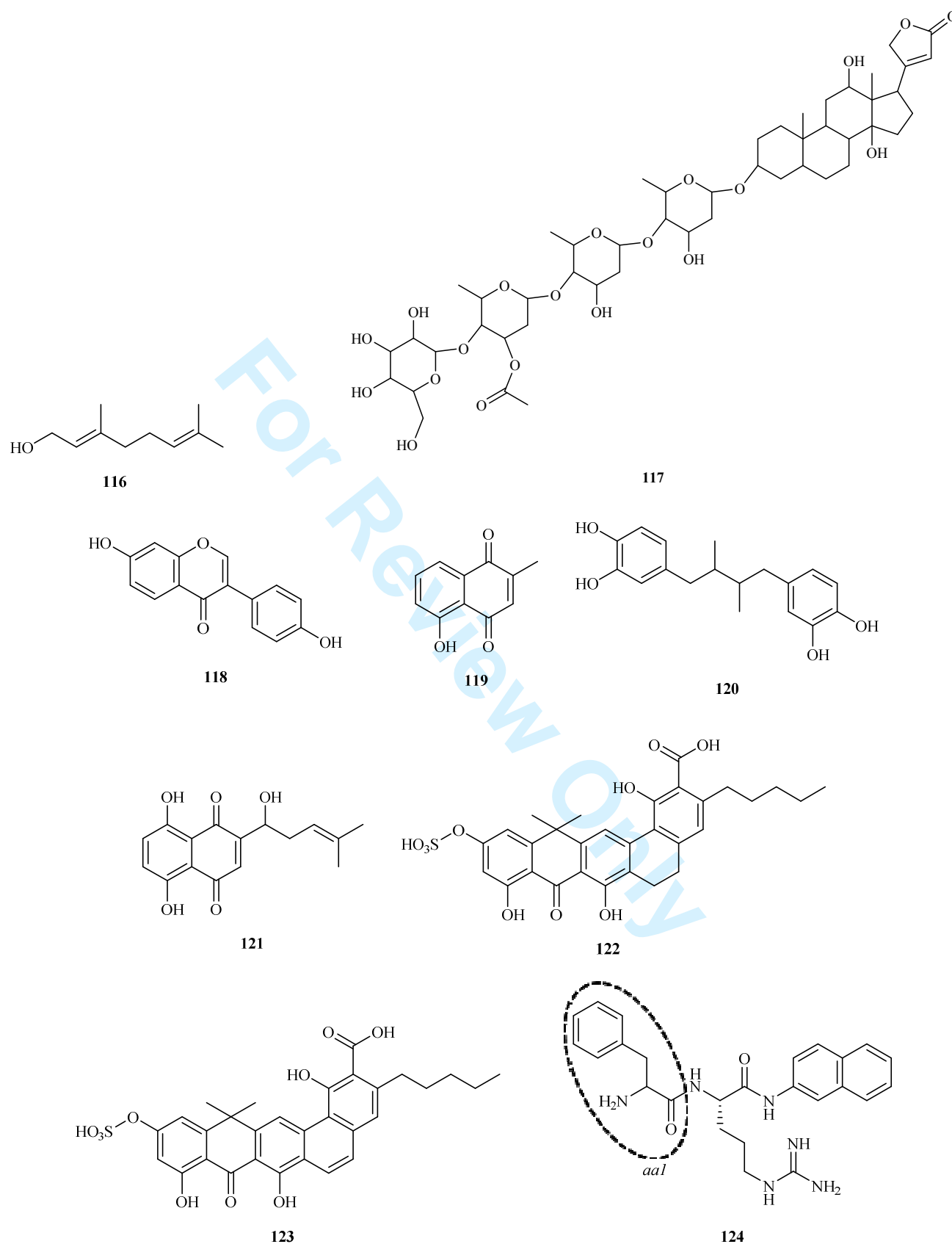


Figure 13 Structures of RND inhibitors 116 – 154. Boc = *tert*-Butyloxycarbonyl.

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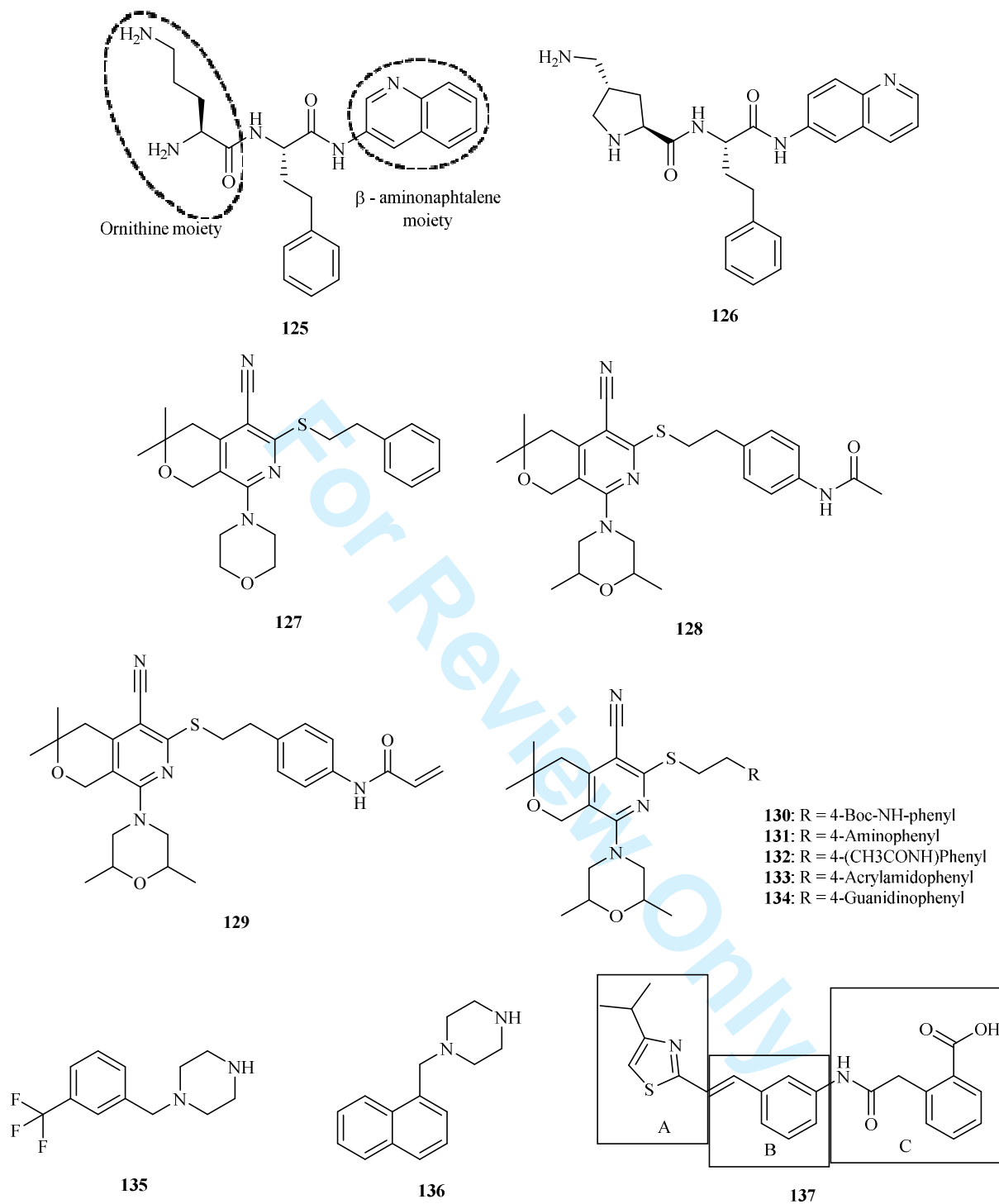


Figure 13 (cont.)

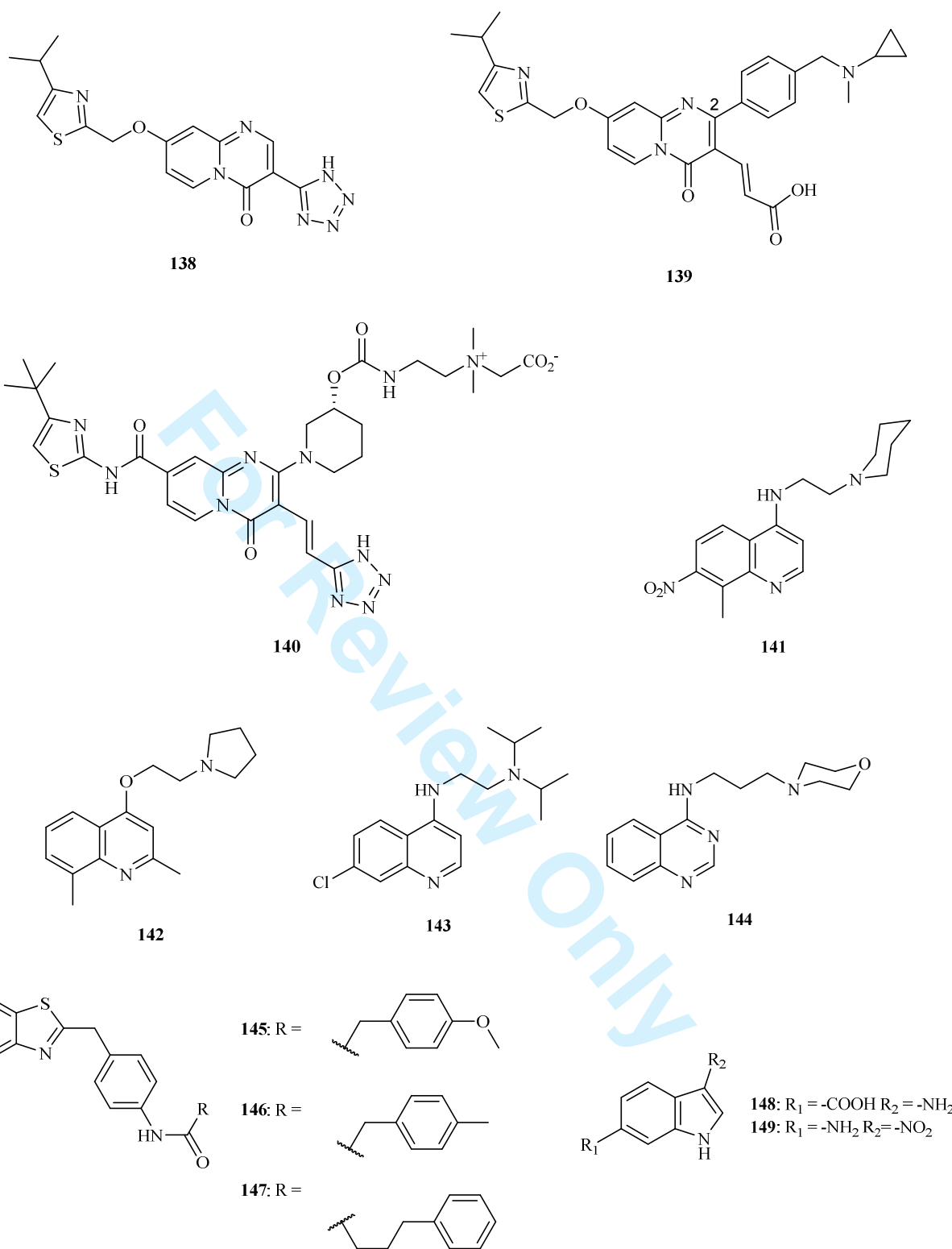


Figure 13 (cont.)

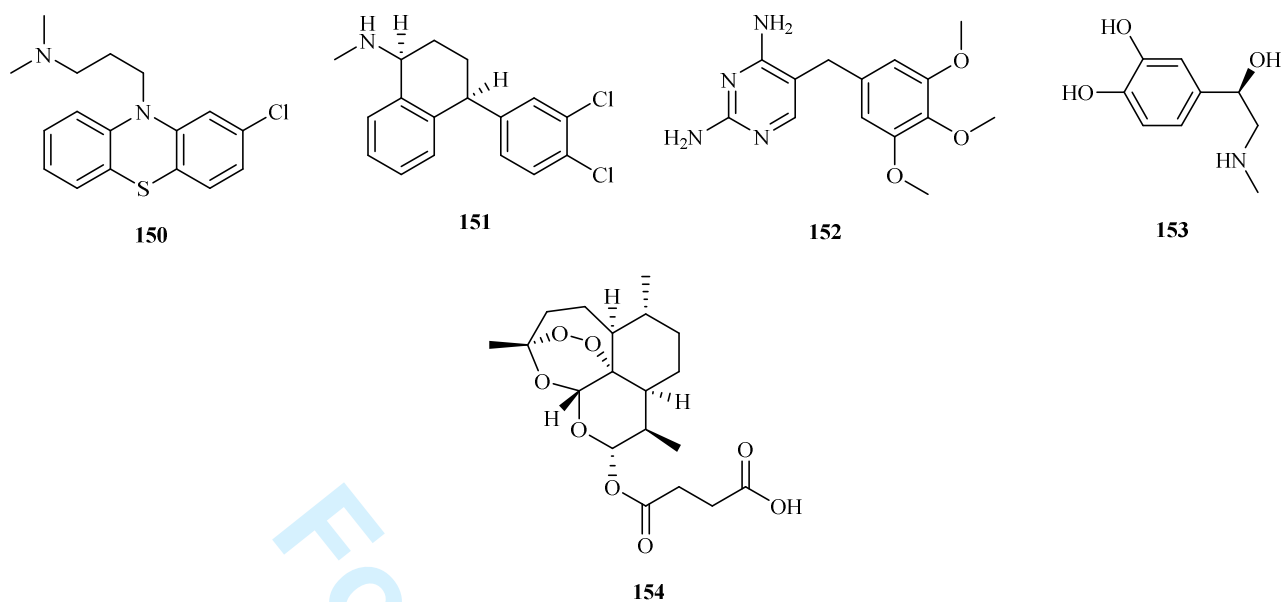


Figure 13 (cont.)

significant restoration of sensitivity to chloramphenicol by 16-fold, and rendered a total sensitivity of the organism when combined with of phenyl-arginine β -naphthylamide (PA β N), which will be discussed further ahead [135].

A study published by Aparna *et al.* (2014) had the aim of identifying natural compounds from plants that present efficacy in potentiating effects of antibiotics in *P. aeruginosa* overexpressing MexAB-OprM and *E. coli* overexpressing AcrAB-TolC through *in silico* virtual screening and pharmacophore approaches [136]. The compounds with the best *in silico* results were then tested for their EPI activity. The two compounds that showed the best activity in potentiating the activity of carbenicillin and levofloxacin in *P. aeruginosa* and *E. coli* were lanatoside C (**117**) and daidzein (**118**). Lanatoside C (**117**) is cardiac glycoside that inhibits the Na⁺-K⁺-ATPase, which can also be the explanation for its EPI activity against MexB and AcrB. Daidzein (**118**) is an isoflavone that has previously showed slight EPI activity in *Mycobacterium smegmatis* and as a modulator of P-glycoprotein, in human cervical carcinoma KB-V1 cells [136].

An *in silico* study by Ohene-Agyei *et al.* (2014) identified five phytochemicals that could be docked similarly to PA β N in the binding pocket of AcrB. Of these five, three of them were able to decrease resistance to antibiotics by inhibiting the AcrAB-TolC system: plumbagin (**119**) increased sensitivity to erythromycin, chloramphenicol, and tetraphenylphosphonium; nordihydroguarectic acid (NDGA) (**120**) potentiated the activity of erythromycin, chloramphenicol, tetraphenylphosphonium, novobiocin, and tetracycline; and shikonin (**121**) decreased resistance to tetraphenylphosphonium [137].

A study proved the usefulness of curcumin (**64**) as a RND EPI at a concentration of 50 μ g/ml, increasing the sensitivity of carbenicillin, ceftazidime, and meropenem in *P.*

aeruginosa, in decreasing order. It also managed to circumvent the resistance to gentamicin and ciprofloxacin in resistant isolates, which PA β N failed, leading to the assumption that curcumin inhibits efflux pumps differently from PA β N [138].

A screening of 85 000 microbial fermentation extracts derived from 3 600 strains of actinomycetes and 3 500 strains of fungi was made, with the aim of discovering EPIs that increased sensitivity of levofloxacin in *P. aeruginosa* overexpressing the MexAB-OprM or the MexEF-OprN pump. Two compounds, EA-371 α (**122**) and EA-371- δ (**123**), produced by a strain of *Streptomyces* sp., demonstrated inhibitory activity against MexAB-OprM [139].

4.1.2. Synthetic Compounds

The first compound with potent inhibition of RND efflux pumps was PA β N (**124**), a dipeptide amine. This dipeptide was identified as a hit compound from the screening of 200 000 samples of small molecules that potentiate the activity of antibacterial levofloxacin against strains of *P. aeruginosa* that overexpressed MexAB, MexCD and MexEF pumps [28, 140]. Compound **124** is a peptidomimetic, developed for using in adjunctive therapy. This compound is a substrate for RND pumps, acting as a competitive inhibitor of multidrug efflux systems, such as MexB, MexD, and MexF, binding to the substrate pocket, impeding antibiotic binding and/or extrusion. Particularly, this EPI binds in a location close to the antibiotic binding site, generating steric hindrance, making it more difficult for the antibiotic to bind. In other words, less quantity of antibiotic is exported, making its intracellular levels higher, thus restoring antibiotic sensitivity [11, 25, 27-28, 141-143]. However, **124** can affect the integrity of the membrane when used in high

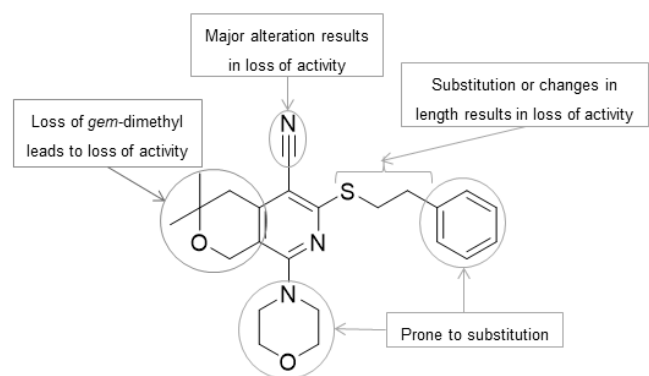


Figure 14 Conclusions from SAR studies for MBX2319 (127) (adapted from [28]).

concentrations, making it prone to the emergency of resistant profiles, namely the modification of the lipopolysaccharide structure, leading to changes in drug penetration [24].

PA β N (124) has been validated against the AcrAB-TolC in *Klebsiella pneumoniae*, *E. coli*, *Salmonella enterica* serovar Typhimurium and *Enterobacter aerogenes*, and in multiple homologous systems including *Acinetobacter baumannii*, *Campylobacter jejuni*, and *Campylobacter coli* [11, 142]. Its potency has been demonstrated against the norfloxacin resistance conferred by the Mex efflux system of *Pseudomonas aeruginosa*, the AcrAB efflux systems of the Enterobacteriaceae, and the erythromycin efflux system of *C. jejuni* [19].

Studies of SAR demonstrated that the basic middle amino acid, arginine, was detrimental for activity, although a substitution with L-lysine showed the same potency as the lead, and the introduction of L-ornithine, originating compound L-Phe-L-Orn- β -Na, showed a 2-fold increased potency. As the ornithine moiety provided a simpler synthesis, it became the standard middle residue in successive compounds [25, 142, 144]. However, these compounds were not stable upon incubation with human serum, due to the occurrence of cleavage of peptide linkage between both amino acids, which is explained by the fact that natural (L) amino acids are contained within its structure. In order to overcome this problem, methylation of the NH that links both amino acids was achieved, maintaining potency and being this derivative able to resist serum proteases. Serum stability was also increased by replacing the L-amino acid with D-amino acid [25, 142]. In the same study, the authors also proved that the amino acid in the *aal* position (Figure 13, 124) should be appropriately substituted, and the replacement of phenylalanine with a homophenylalanine led to improved potency. The problem with this compound was the fact that the ornithine moiety could easily form a lactam, rendering the compound inactive. This was overcome by switching the L-ornithine with the L-phenylalanine, which originated L-Orn-L-hPhe- β -Na, which originated a compound just as potent, but with no propensity to form a lactam. As for the β -aminonaphthalene moiety, it was proven that replacing it with 3-aminoquinoline led to

reduced cytotoxicity and intrinsic antibacterial activity, even though its potency was slightly reduced [25, 142]. These efforts originated the compound L-Orn-L-hPhe-3-NHQ (125). In conclusion, the analogues synthesized by Renau *et al.* (1999) did not show improvements in potency when compared to PA β N (124), but showed increased stability [142].

The same group developed further studies with this type of molecules and demonstrated that conformational restriction of the amino group of ornithine showed less toxicity, while maintaining potency. Such compounds were attained by adding a proline substituent, thus incorporating the two basic residues necessary for activity, which resulted in the successful synthesis of constrained derivatives, more potent and less toxic than 125. The most interesting derivative, compound 126, was as potent as 125, but more than 4-fold less toxic. This compound also retained the activity against RND efflux pumps MexAB-OprM, MexCD-OprJ and MexEF-OprN. Although this compound showed similar protein binding in rats as 125, its pharmacokinetic parameters were improved, and its efficacy in combination with levofloxacin was demonstrated in an *in vivo* model [145].

In a latter study developed by the same group, several non-peptide analogues were synthesized, and it was hypothesized that the peptide backbone is not essential for the activity of this type of compounds. The results suggested that the inhibition of pumps was possible due to the di-cationic nature of the compound, the appropriate lipophilicity and a disposition similar to that of compound 125 [146].

MBX2319 (127) is a pyranopyrimidine EPI inhibitor of AcrAB-TolC efflux pump in *E. coli* and other Enterobacteriaceae [141]. Compound 127 did not show any antibacterial activity, which is a positive feature for EPIs. It also potentiated the antibacterial activity of fluoroquinolones, such as ciprofloxacin and levofloxacin, and β -lactams, such as piperacillin against strains of *E. coli* that presented AcrAB-TolC efflux pumps [28, 141]. In *P. aeruginosa*, although compound 127 presents activity against RND-type pumps, it is unable to penetrate the outer membrane, which is highly selective [28].

In *E. coli*, the target of compound 127 is the membrane transporter AcrB. This molecule binds to the hydrophobic trap of the T protomer, interacting with the hydrophobic residues that constitute the deep binding pocket as a hydrophobic trap. π - π Interactions also occurs, between the pyridine ring of 127 and the aromatic side chain of Phe-628. The phenyl and morpholinyl groups also interact with Phe-178 and Phe-615. The dimethylenesulfide linker and the geminal dimethyl group present van der Waals interactions between the side chain of Phe-178, and Tyr-327 and Met-573, respectively [28, 147].

SAR studies demonstrated that the maintenance of activity depended on the geminal dimethyl moiety of the tetrahydropyran, the nitrile group and the length of the dimethylenesulfide linker. This data is presented in Figure 14. On the other hand, the morpholine moiety and aryl group are both prone to substitution, originating more stable

derivatives in terms of activity, solubility, and metabolism [28]. Studies with this scaffold have shown that non-acidic substituents can be added to the phenyl group, in order to improve potency and CYP450 inhibition, and modifications to the morpholinyl group has greater effects on solubility and stability [140].

Analogues were synthesized by Sjuts *et al.* (2016), by changing the structure and functional groups of compound **127** (148). They reached the conclusions that the introduction of a 2,6-dimethyl to the morpholinyl group improved both microsomal stability and EPI activity. The replacement of the morpholinyl group with 2-methoxyethylpiperazinyl improved aqueous solubility, at the cost of a slight reduction of EPI activity. Two analogues, **128** and **129**, resultant of the combination of 2,6-dimethylmorpholinyl and acetamide or acrylamide, respectively, on the phenyl group, resulted in a 10-fold to 20-fold increase in EPI activity against *E. coli*, respectively, comparing to MBX2319 (**127**) [28, 148]. The increased EPI activity of these compounds has its basis in the acetamide and acrylamide groups and the morpholinyl group. The acetamide groups are engaged in highly ordered and complex hydrogen bonds, with its center in a solvent water molecule, which plays the role of a hydrogen bond donor to the carbonyl backbone oxygen of Ala-286 and Gln-151 side chain of AcrB. The acrylamide group forms a bridging hydrogen bond through a water molecule to the side chain of Gln-176 [28].

Nguyen *et al.* (2015) also synthesized a series of analogues, with the morpholine scaffold substituted by a 2,6-dimethylmorpholinyl group (140). Many of these analogues exhibited a solubility improvement of 10-fold, compared to compound **127**. The most potent and promising compounds of these series were compounds **130**, **131**, **132**, **133** and **134** [140].

Through a screening of an *N*-heterocyclic organic compound library, Bohnert and Kern (2005) were able to find a novel class of EPIs, the arylpiperazines [25, 149]. The aim of their work was to find compounds that would potentiate levofloxacin potency against strains of *E. coli* overexpressing *acrAB* and *acrEF*. This was fulfilled when they found phenylpiperazine derivatives with promising activity [25, 149]. SAR studies suggested that elongation of the spacer between the benzene ring and the piperazine ring would enhance potency. Also, substitutions on the benzene ring by halogen atoms led to an increase in potency [24, 149]. On the other hand, the potency would decrease when an ethyl or phenyl group was added to the piperazine ring. With this, the authors found the most potent phenylpiperazine, *meta*-TFMPP (**135**), and naphthylpiperazine, 1-(1-naphthylmethyl)-piperazine (**136**) [149].

NMP (**136**) was the most potent unsubstituted arylpiperazine, able to increase the intracellular concentration of chloramphenicol, tetracycline, linezolid, fluoroquinolones, and macrolides. Although it does not display action in *P. aeruginosa*, it shows effectiveness in *A. baumannii* and several Enterobacteriaceae [11, 23]. EPI

activity was also reported in clinical isolates of *E. coli*, most relevantly in fluoroquinolone resistance [150]. Through computational analysis, it was found that NMP (**136**) binds with high affinity to the lower part of the deep pocket of the B monomer, overlapping in part the binding site of chloramphenicol [11]. The binding site for NMP (**136**) includes interactions with hydrophobic residues near the hydrophobic patch and Gly-617 of the G-loop. This data suggests that NMP (**136**) interferes with the movement of the G-loop, important for extruding substrates, thus inhibiting the action of AcrB [25].

Nakayama *et al.* (2003) performed a series of syntheses in order to attain a compound specific for the inhibition of a MexAB-OprM specific EPI [151-152]. They identified a hit, compound **137**, through high-throughput screening for levofloxacin potentiation using a strain of *P. aeruginosa* strain overexpressing MexAB-OprM. The features this group was looking for was the effective inhibition of efflux pump and the lack of intrinsic antibacterial activity, since efflux pumps are not vital for the organism's survival [151-152]. It was shown that this compound effectively potentiated the activity of levofloxacin, leading to an 8-fold decrease in the antibiotic's MIC. However, its physicochemical properties were problematic: water solubility was poor and it presented high affinity to serum albumin. Therefore, they divided the molecule into three parts, A, B, and C (**137**) and looked for alternative scaffolds and substituted polar groups, in order to reduce serum protein binding [152].

It was shown that when a carboxamide was introduced in A, the affinity to serum albumin was diminished, as was its activity. More polar moieties led to a complete loss of activity. The strategy for the styrene portion (B) that was the most successful was to substitute the benzene ring to a 3,5-disubstituted pyridine, which had both good activity and no major serum influence. As for the vinyl group in B, it was demonstrated that molecules bearing methoxy and ethylene moieties displayed favorable activity profiles. As for part C, the basis of the modifications was the fact that the introduction of bulky or hydrophilic groups adjacent to the carboxyl group would reduce protein binding. An enantiomeric pair containing a dioxolane moiety presented both activity and serum stability, being the *S* enantiomer more active [152].

Regarding the general scaffold, new compounds were designed, keeping in mind that the thiazole moiety was crucial for activity. Therefore, a molecule having a thiazole side chain attached to the C-7 position of a quinolone was synthesized. Substitution with a tetrazole portion yielded better results than substitution with a carboxylic acid. One compound (**138**) presented the best activity and serum stability [153].

Further efforts were placed in order to increase the potency of this compound. The *in vitro* activity was enhanced by the introduction of a hydrophobic group at the 2-position of the pyridopyrimidine scaffold, and hydrophilic substitution would not compromise the activity. The most promising compound had the inclusion of an olefin spacer between the tetrazole and the pyridopyrimidine scaffold, with the

limitation of being prone to photoisomerization [154]. To circumvent this, the ethylene tether between the pyridopyrimidine was replaced with an amide bond, providing stability against photoisomerization and, unexpectedly, improving potency. However, another problem arose: the lack of solubility for intravenous use [155]. The following step was the introduction of a secondary amine, giving a zwitterionic analogue, with retention of activity. C-2 Analogues, namely with cyclic substituents, also showed promising results [156]. Once again, the problem of solubility arose. Therefore, efforts were placed in making the molecule more hydrophilic, with the introduction of substituted aromatic moieties. Analogues 4-substituted were the most potent, and the introduction of a morpholine moiety led to increased solubility, maintaining the serum stability. Compound **139** was the most balanced molecule in terms of the desired features [157]. The last refinement made was the introduction of a quaternary ammonium salt, which led to D13-9001 (**140**). Adding to its high solubility and safety, it was able to potentiate the activity of aztreonam [158]. **Figure 15** illustrates the SAR established for **140** and derivatives.

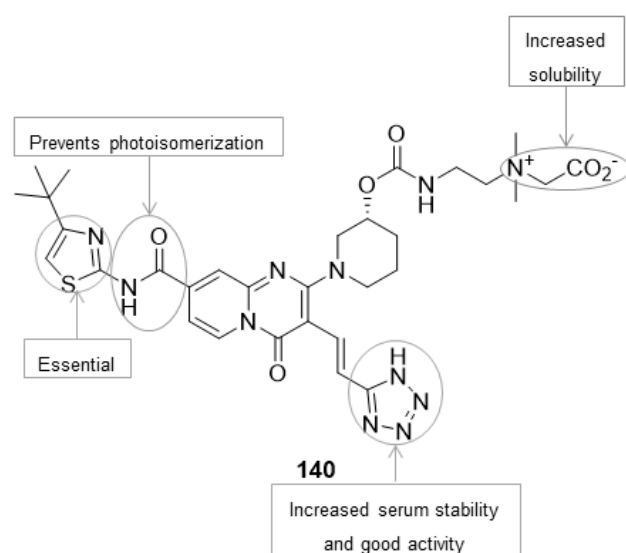


Figure 15 Conclusions from SAR studies for pyridopyrimidine and analogues.

3D Crystal structures of **140** bound to the target, either AcrB or MexB, show that the *tert*-butyl thiazolyl aminocarboxyl pyridopyrimidine moiety of this compound binds tightly to a narrow depression, designated by hydrophobic trap, close to the deep substrate binding pocket. Additionally, the tetrazole ring and the piperidine acetoamino ethylene ammonioacetate moiety interact with ionic and/or hydrophilic residues in the substrate translocation channel. The piperidine acetoamino ethylene ammonioacetate moiety also covers part of the minocycline and doxorubicin binding site [23, 28, 151].

Quinoline derivatives with EPI activity include a multiplicity of derivatives, including pyrrodo-, alkoxy-, thioalkoxy-, alkylamino- and chloro- derivatives. These compounds were able to restore an intracellular concentration of antibiotic drugs expelled by efflux pumps, as well as to induce the increase in antibiotic susceptibility, acting as competitive inhibitors of the antibiotic flux. In *E. aerogenes* and *K. pneumoniae*, quinolines were more effective than PA β N (**124**) in inhibiting the activity of the AcrB pump, potentiating the activity of fluoroquinolones, tetracycline, and chloramphenicol [11, 24, 26]. Among the different classes of substituted quinolines, it was proven that the best side chain was piperidinoethyl, which potentiated efficiently the restoration of drug susceptibility in alkoxy- and thioalkoxyquinolines. The heteroatom connecting the side chain is also crucial for activity, being the most potent the amino substituent, then the thioalkyl-, followed by the oxo-derivative. The position of the branched substituted groups is also detrimental for the EPI activity [24]. Branched side chains containing piperidinoethyl and morpholinopropyl groups were associated with the most interesting results, being compound **141**, containing a piperidinoethyl side chain, the most active when in combination with chloramphenicol [159]. It has been hypothesized that the pump inhibition is greatly influenced by the branched side chain, and takes place either in the inner-membrane transporter or at the junction between the inner pump and the outer channel [159].

Given these promising results, further research in this kind of compounds was performed. Alkoxyquinolines were synthesized and tested against a strain of *E. aerogenes* overexpressing the AcrAB efflux pump. Compound **142** induced an increase in the susceptibility of chloramphenicol, tetracycline, and fluoroquinolone. This could happen due to interference during active pumping out of the antibiotic [160].

Chloroquinolines also demonstrated efficacy as EPIs, modulating chloramphenicol activity, decreasing its MIC 8-fold for resistant strains. Synthesis of analogues led to the conclusion that amino derivatives are more potent than thiol derivatives, pointing out the importance of the side chain for the efflux inhibition. Protonation of the nitrogen could also play an important role at environmental pH, stimulating recognition and binding of the drug to the sites located within the pump's cavity. The heteroatom is also crucial for the activity, being nitrogen the most efficient. Compound **143** is the chloroquinoline with the best results found for the inhibition of the main efflux pump of *E. aerogenes* [161].

Alkylaminoquinazoline derivatives lacking a nitro group were also evaluated for their ability to decrease chloramphenicol and quinolone efflux in *E. aerogenes*, through the inhibition of AcrAB efflux pump. The results obtained suggest that this family of compounds recognizes the same pump site responsible for the transport of chloramphenicol and quinolones, competing with them. Structurally, it has been shown that a morpholine group with a propyl chain makes a more active alkylaminoquinazoline.

The most active compound of this family is compound **144** [162].

Benzothiazoles 2-substituted were first tested for the potentiation of ciprofloxacin in AdeABC overexpressing *A. baumannii*. These compounds did not show antibacterial activity when tested alone in bacteria. However, when combined with ciprofloxacin, there was an observation of a reversal in antibacterial susceptibility. The synthesis of several derivatives of this family led to pharmacophore studies, regarding the features of the most promising compounds. Using computational methods, it was revealed that, in order for the compounds to be biologically active, the following features should be present: the nitrogen atom in the thiazole ring and the carbonyl oxygen in the amide function substituted on the second position of the benzothiazole ring must have an hydrogen bonding acceptor property; the benzene ring in the fused ring system, the phenyl group attached to the second position of the benzothiazole ring and the phenyl ring in the 2-phenylacetamide and/or 3-phenylpropionamide moiety are essential, since they provide a hydrophobic aromatic property. Of the synthesized compounds, the ones that gathered these features and, therefore, presented the most promising activity, were compounds **145**, **146**, and **147** [163]. These compounds were also tested for their activity against AcrAB-TolC, in an overexpressing strain of *E. coli*, reducing the MIC of ciprofloxacin 10-fold, in the case of **146** and **147**, and 8-fold, for **145** [164]. Unlike AdeABC, AcrAB-TolC's crystal structure is elucidated, making docking studies possible. This predicted that all three compounds act as AcrB substrates, binding to the distal pocket site in the AcrB porter domain by blocking or inhibiting the ciprofloxacin binding site, generating steric hindrance and thus impeding the binding of the antibiotic. Furthermore, **146** and **147** showed stronger binding interactions than ciprofloxacin [164].

Indole derivatives have been useful in inhibiting protein targets of virus, tumors and bacteria. Since small molecules with conjugated aromatic rings, in which indoles fit, present high potential as EPIs, efforts were placed in synthesizing derivatives that presented this kind of activity. The TolC 3D structure has already been disclosed, making possible the structure-based design of inhibitors. Indoles **148** and **149** were proven efficient inhibitors of TolC in *E. coli*, potentiating the effects of chloramphenicol, tetracycline, erythromycin, and ciprofloxacin, with decreased MIC values of 2-fold for tetracycline and erythromycin and 8-fold for the other two [165].

4.1.3. Existing Drugs

Other drugs used for treatments other than antibacterial have demonstrated EPI activity. Tetracycline resistance in *P. aeruginosa* was reduced 8-fold when the phenothiazine fluphenazine, an antipsychotic, was present. Chlorpromazine (**150**) also showed EPI-like activity for the BpeAB-OprB system in *Burkholderia pseudomallei* and a reduction of the expression of *acrB* in *E. coli* and *S. enterica* samovar

Typhimurium, suggesting an inhibition of AcrB production [11, 27, 128].

Selective serotonin-reuptake inhibitors have been studied for their ability to act as EPIs for RND efflux pumps in *E. coli*. It was found that sertraline (**151**) was capable of potentiating the activity of fluoroquinolones. However, its activity is limited, since it is possible that this compound also acts as an inducer of efflux pumps. Studies have shown that this compound could accumulate in tissues or cells, and therefore, sertraline's (**151**) concentration may be higher than that measured in plasma. This means that a standard dose of this drug could enhance antibiotic efficacy. It has not yet been studied if a sertraline (**151**) metabolite could be administered at a dose higher than sertraline. However, it was hypothesized that it could have a better EPI activity, while causing less undesired effects on the central nervous system [166].

Through the observation of previously reported EPIs, Piddock *et al.* (2010) suggested that simple heterocyclic nitrogen-containing compounds could also act as EPIs and synergize with ciprofloxacin in strains of *S. enterica* serovar Typhimurium that overexpressed the AcrAB-TolC efflux pump [167]. It has been shown that compounds containing a phenylethylamine moiety, as PAβN (**126**) does, such as epinephrine, norepinephrine, and cathinone, are able to potentiate the activity of ciprofloxacin. Of the compounds tested, trimethoprim (**152**) and epinephrine (**153**), showed the best results for the tested strains, which included, apart from *S. enterica* serovar Typhimurium, *P. aeruginosa*, *E. coli*, and *K. pneumoniae*. These compounds showed synergy for the strains where AcrAB-TolC was active, but showed no activity when the pump was inactive. Thus, it can be hypothesized that these compounds either inhibit the expression of the genes encoding AcrAB-TolC, or interact directly with the pump [167]. Both compounds possess structural features similar to previously described EPIs, such as PAβN (**124**), L-Orn-L-hPhe-3-NHQ (**125**), and NMP (**136**), such as an aromatic ring linked to a basic nitrogen, in the case of epinephrine (**153**), or a pyrimidine ring, for trimethoprim (**152**). Trimethoprim (**152**) is already used in combination with another antibiotic, sulfamethoxazole, and combination with a quinolone was hypothesized to be a viable choice. On the other hand, a combination of epinephrine (**153**) with an antibiotic does not seem a viable option, because of the likelihood of the occurrence of sympathomimetic effects [167].

The antimalarial artesunate (**154**) has also shown EPI activity, enhancing the effects of β-lactam antibiotics, such as penicillin G, oxacillin, and ampicillin, and novobiocin against *E. coli*. The mechanism through which **154** expresses its activity is by the inhibition of the expression of the AcrAB-TolC system, not exhibiting antibiotic activity by itself [168].

5. SMR

The SMR family comprises the smallest drug efflux proteins known, possessing only four transmembrane segments in its

composition, and are exclusive to bacteria. They are involved in the efflux of lipophilic compounds, such as quaternary ammonium salts and a variety of antibiotics. These pumps also use an electrochemical proton gradient in order to exert its efflux activity [7, 32, 38, 169].

One example of a SMR-type pump is the EmrE transporter (Figure 16, PDB 3B61), present in *E. coli*. In the absence of the ligand, the asymmetric unit of EmrE contains eight EmrE monomers, with four transmembrane helices composing each of them. If a ligand is present, it can function as an inverted homodimer [7, 170].

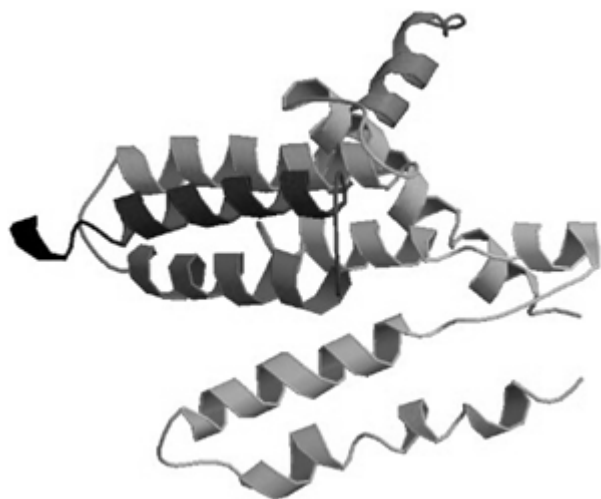


Figure 16 Crystal structure of EmrE (PDB 3B61) of *E. coli*.

There have not been many studies concerning inhibitors of this family of efflux pumps, and the few that have been performed were only *in silico*. Figure 17 shows the structures of the SMR inhibitors that will be further discussed.

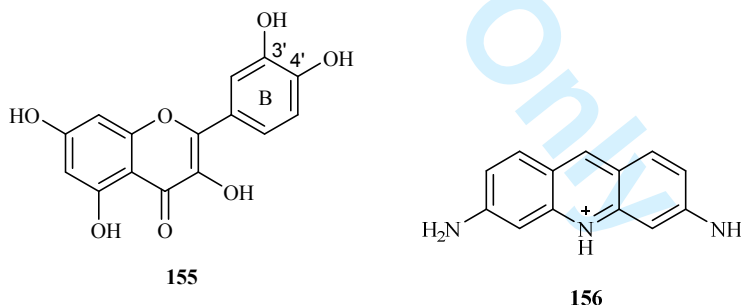


Figure 17 Structures of SMR inhibitors 155 and 156.

5.1. SMR inhibitors

5.1.1. Natural Products

Quercetin (155) is a flavonol, present in many plants. This compound is very well tolerated in humans, as are its glycoconjugates, and presents a multiplicity of activities, such as antioxidant, anti-inflammatory and antimycobacterial. Only docking studies of this compound

with two pumps belonging to the SMR family, the Mmr, in *Mycobacterium smegmatis*, and the EmrE pumps, in *E. coli*, were carried out. In Mmr, the hydroxyl groups present in the 3' and 4' positions of the B ring of quercetin were predicted to interact with Glu-14 in the helix 1, and the keto group of the hydroxychromen ring of the ligand interacts with the Trp-48 of the helix 1. Additionally, hydrophobic interactions were shown by the residues Phe-44, Tyr-40 and Trp-48, and π - π interactions were found with Phe-44. Furthermore, 155 was also predicted of interacting with dimeric forms of the protein [171]. In the case of EmrE, the interaction between the 3' and 4' hydroxyls with Glu-14 can also be observed, as can the hydroxyl in the C-7 position with Tyr-60. Hydrophobic interactions are present, with the residues Ile-11, Ser-64, Gly-67 and Leu-70, and π - π interactions exist with the residues Tyr-60 and Trp-63. Structural studies suggest that EmrE is mostly in the dimer state, which also interacts with quercetin (155) [171].

5.1.2. Synthetic Compounds

Cation ligands were subjected to docking studies with the Mmr protein of the SMR family in *Mycobacterium tuberculosis* and the EmrE dimer from *E. coli*. The rationale behind this study is the fact that SMR can extrude cations. This study led to the identification of new virtual leads, amongst which is compound 156. ADME studies prove that all the molecules present in this study obey the Lipinski rule of five and the Jorgenson rule of three, which shows that the compounds possess good drug-like properties [172].

6. MATE

MATE transporters are responsible for the efflux of cationic,

lipophilic substances, providing bacteria and cancer cells with MDR, using Na^+ or H^+ influx. They have 12 transmembrane helices, constituted by 400 to 700 amino acids. The mechanism of efflux is thought to happen by a rocker-switch mechanism. Members of the MATE family include the MepA (Figure 18, PDB 1TZZ) transporter, in *S. aureus*, even though seventeen MATE proteins have been identified in eleven species, capable of extruding antibiotics. Ethidium bromide, tetraphenylphosphonium, acriflavine,

berberine, and norfloxacin have been reported as MATE substrates [38, 173-181].

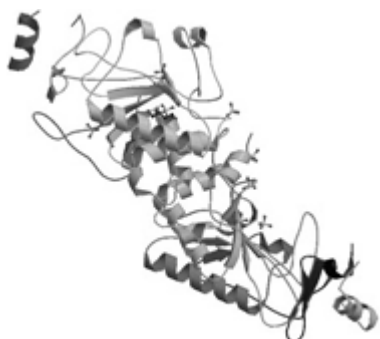


Figure 18 Crystal structure of MepA (PDB 1TZP) of *E. coli* in its inactive form.

In **Figure 19** are compiled the structures of the compounds that have so far proven their usefulness as MATE inhibitors.

6.1. MATE inhibitors

6.1.1. Natural Products

A study performed by Kesharwani *et al.* (2017) accomplished the docking of phytochemicals with the NorM transporter, belonging to the MATE family [182]. After a screening of a library of natural products, three hits were identified: derhamnosyl suspensaside (**157**), prunin 7''-*O*-gallate (**158**), and quercetin diglucoside (**159**). These were the best compounds, as their results in binding free energy calculation were the most favorable. When binding free energy decomposition and hydrogen and hydrophobic statistics were taken into account, **158** was elected the best hit compound. Furthermore, this compound was stable during the simulation. Overall, it has been suggested that the compounds which interact towards the cation binding residue in the central cavity of NorM will have better results, and that good inhibitors should have interaction with a few key residues, Gln-34, Val-35, Gly-38, Phe-63, Tyr-67, Ile-68, Glu-261, Tyr-294, and Asp-377, which will lead to

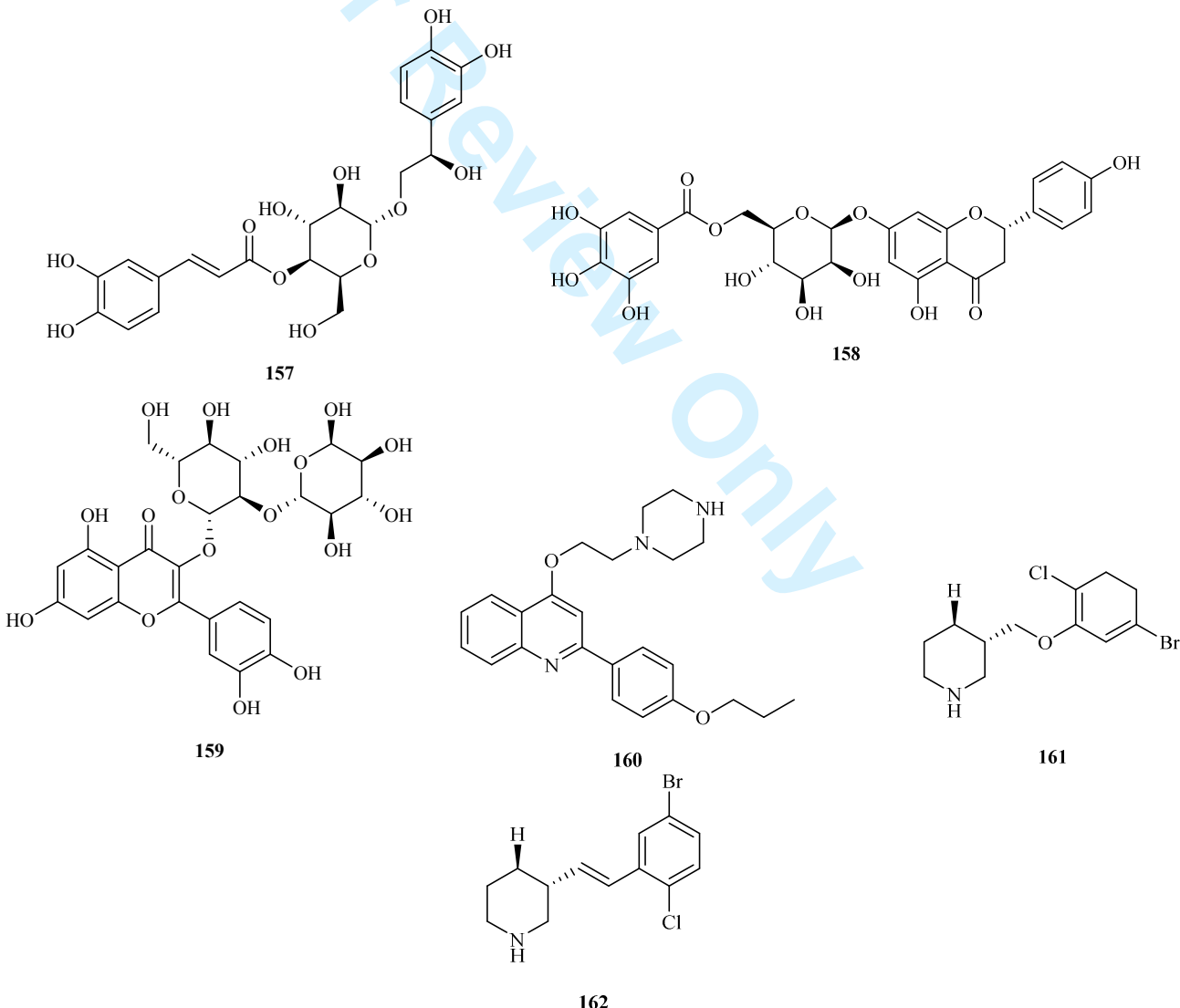


Figure 19 Structures of MATE inhibitors **157** – **162**.

maximum stability and affinity with the NorM transporter [182].

6.1.2. Synthetic Compounds

These compounds have been previously described as MFS inhibitors, namely of the NorA pump. However, similar activity has been described for the MepA pump, of the MATE family. Compound **80** (Figure 5), incorporating a bisaryl urea moiety at the 7-position, was able to successfully inhibit approximately 84% of ethidium bromide efflux in a MepA overexpressing strain of *S.aureus* [104].

Phenylquinoline derivatives also proved efficient against the same strain. Differently from what was observed with NorA, the compounds that inhibited MepA most efficiently were **86** (Figure 5) and **160** (Figure 19), which were able to reduce ethidium bromide's MIC by 16-fold at concentrations of 0.78 and 1.56 $\mu\text{g/ml}$, respectively, in the MepA overexpressing strain. Ethidium bromide was chosen, instead of ciprofloxacin, as it is a better MepA substrate and other derivatives were able to inhibit both NorA and MepA efflux pumps, being able to completely restore the activity of ciprofloxacin and ethidium bromide against strains overexpressing such pumps [107].

Paroxetine derivatives (**104**), effective against the NorA pump, also demonstrated potency in inhibiting the MepA pump in *S. aureus*. Oppositely to NorA, paroxetine derivatives do not need the fluorophenyl ring at position 4 to be MepA EPIs. In fact, 4-unsubstituted analogues proved to be more potent than 4-F-phenyl derivatives in inhibiting the MepA pump, as is the case of compound **161** and **162** [122].

CONCLUSIONS

Efflux pumps are one of the main causes for antimicrobial resistance. Their inhibition can lead to the restoration of bacteria susceptibility and antibiotic efficacy. Throughout this review, the data presented leads to the conclusion that EPIs are promising compounds and can help reversing the problem of antimicrobial resistance. However, the research into these kind of compounds is still in a very preliminary state, since no EPI is yet under clinical trials.

Even though no general structural features can be pointed out for a molecule to be an EPI, it can be observed that small molecules with high lipophilicity are most likely to fulfill the requirements to inhibit efflux pumps. Hydrophilic compounds can also be transported through porins present in the membrane. Heterocyclic compounds also show potential as EPIs, mainly nitrogen heterocycles, but also fluorine and boron heteroatoms, since the likelihood of bacterial exposure to these atoms is very low. The basis for the MDR pumps inhibition has to do with the penetration rules, and whether or not a compound is capable of penetrating the bacterial membrane. Therefore, a library of compounds that are able to do that would be something that would bring advances to this study field.

One of the main aims of this review is to present SAR studies, so that pharmaceutical chemists can refine molecular

structures, leading to the achievement of more potent and specific compounds. The use of synthetic approaches, either to modify molecules obtained from natural sources, or drugs that are already in therapeutic use, or even to develop novel compounds, is vital for these SAR studies. The search of EPIs derived from natural products comes from the fact that plants need to protect themselves from pathogenic microorganisms, and produce metabolites that act as antimicrobial and EPIs, that synergize with each other. However, the referred compounds are not specific for the pump they have been studied, showing a multitarget character: flavonoids, coumarins, cinnamic acids, among others, present a multiplicity of activities already described, suggesting their promiscuity.

The advantage of assays with existing drugs lies in their safety profile and, most times, their clinical use for combination therapy has been characterized. In some cases, the compounds were even investigated as inhibitors of human efflux pumps, for cancer treatment. This can be regarded as an advantage, leading to a new class of drugs with dual human/bacterial EPI, particularly useful in cancer patients with infections, potentiating the effects of both the anticancer and the antibacterial drugs.

The future in studies in bacterial EPIs should lie either in the search for selectivity for the bacterial target, or in the disclosure of the common features of the universal bacterial EPI, that can successfully target all efflux pumps. The SAR studies gathered herein with the 3D structural information available are expected to accelerate the structure-based design of new EPIs, founded on the existing models.

LIST OF ABBREVIATIONS

3D-QSAR: Three-dimensional quantitative structure activity relationship; ABC: ATP-binding cassette; ADME: Absorption, distribution, metabolism and excretion; ATP: Adenosine triphosphate; BCRP: Breast cancer resistance protein; COX-2: Cyclooxygenase-2; CYP450: Cytochrome P450; EPI: Efflux pump inhibitor; IC₅₀: Half maximal inhibitory concentration; MATE: Multidrug and toxic compound extrusion; MDR: Multidrug resistance; MIC: Minimum inhibitory concentration; MFS: Major facilitator superfamily; MRP: Multidrug resistance protein; MRSA: Methicillin-resistant *Staphylococcus aureus*; NDGA: Nordihydroguarectic acid; NMP: 1-(1-Naphthylmethyl)-piperazine; P-gp: P-Glycoprotein; PA β N: Phenyl-arginine β -naphthylamide; PDB: Protein Data Bank; PSSRI: Phenylpiperidine selective serotonin reuptake inhibitors; RND: Resistance-nodulation-division; SAR: Structure activity relationship; SMR: Small multidrug resistance.

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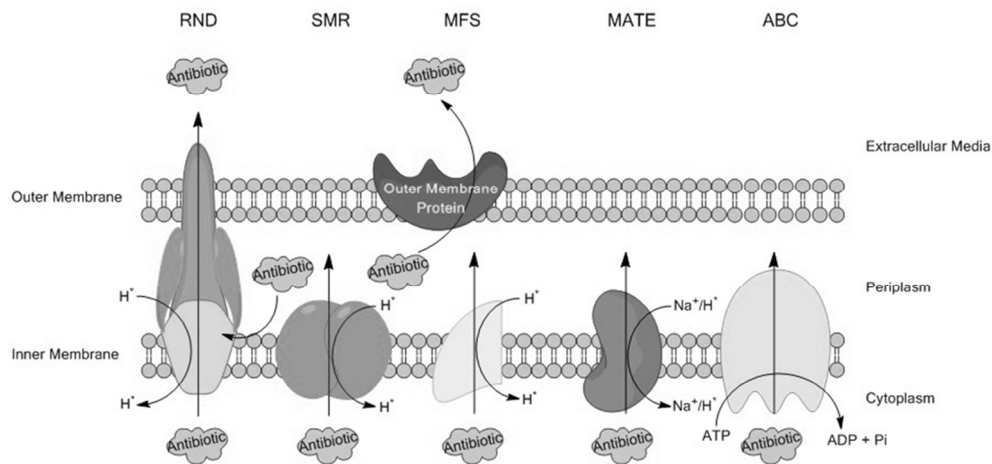


Figure 1 Representation of the five efflux pump families (adapted from [8]).

307x147mm (96 x 96 DPI)



Figure 2 Crystal structure of MsrA, from Mycobacterium tuberculosis in complex with protein-bound methionine (PDB 1NWA).

92x119mm (96 x 96 DPI)



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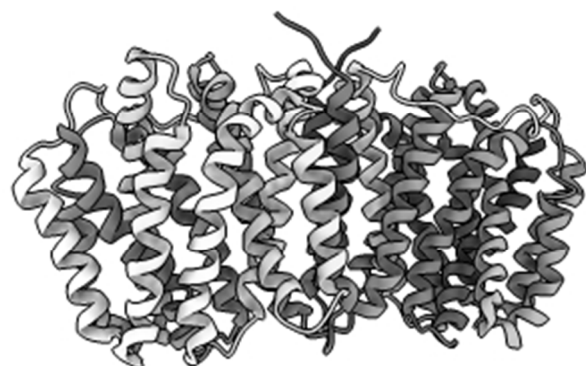


Figure 4 Crystal structure of EmrD, from *E. coli* (PDB 2GFP).

82x54mm (96 x 96 DPI)

Review Only

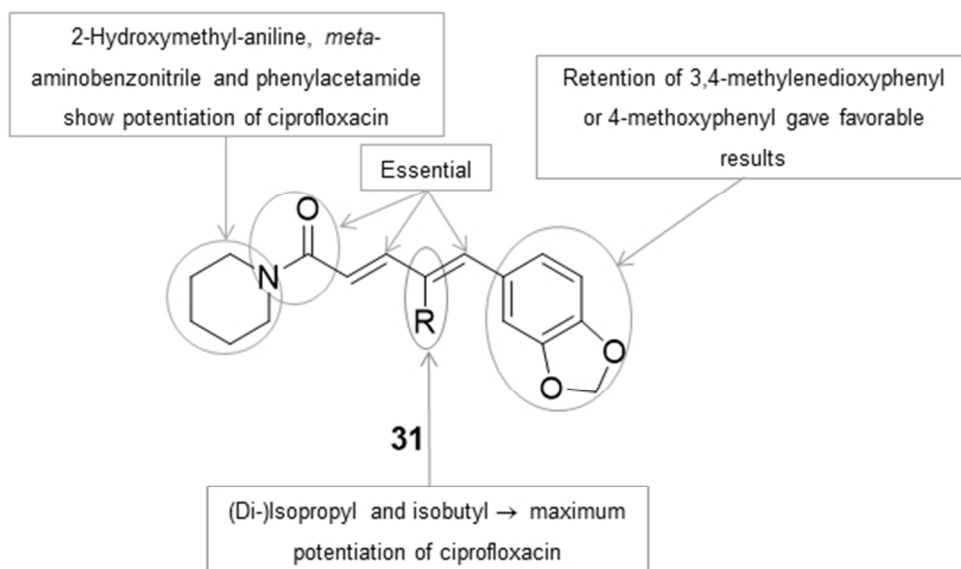


Figure 6 Conclusions from SAR studies for piperine and its derivatives.

156x91mm (96 x 96 DPI)

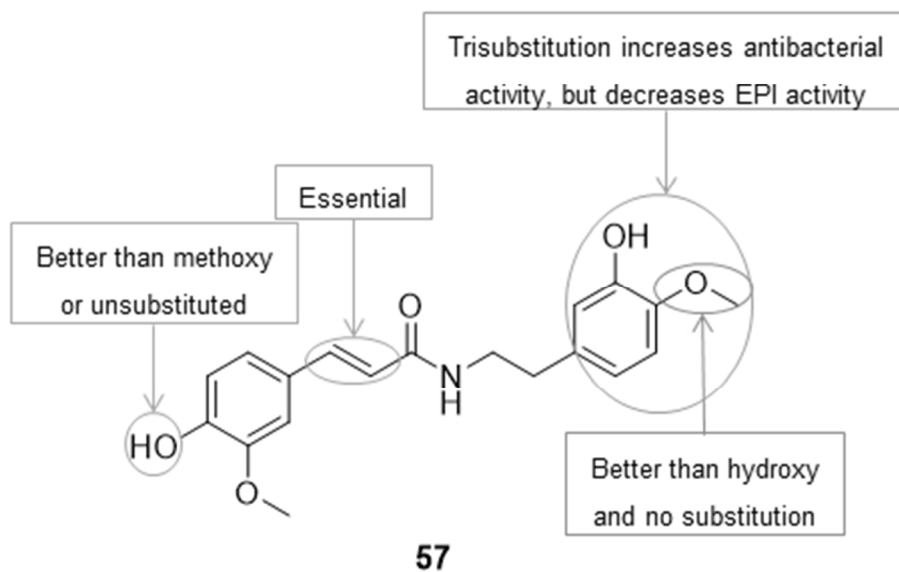


Figure 7 Conclusions from SAR studies for compound 57.

124x80mm (96 x 96 DPI)

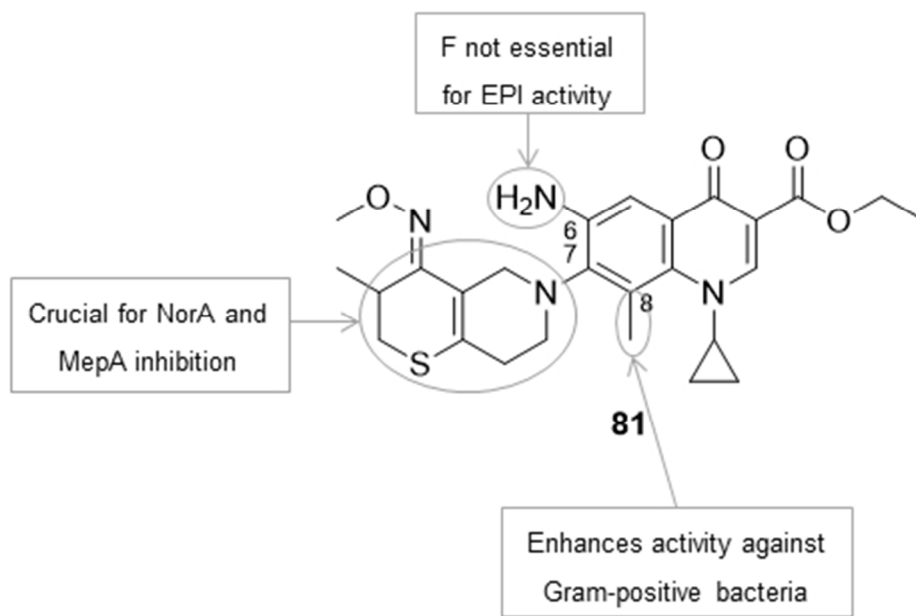


Figure 8 Conclusions from SAR studies for fluoroquinolone 81 derivatives.

124x84mm (96 x 96 DPI)

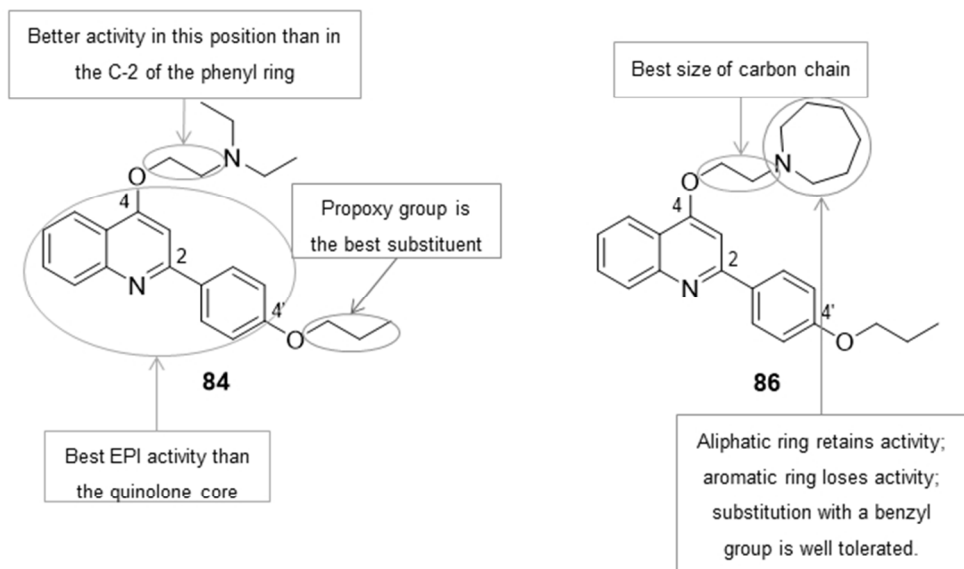


Figure 9 Conclusions from SAR studies for 2-phenyl-4-hydroxyquinoline derivatives 84 and 86.

177x106mm (96 x 96 DPI)

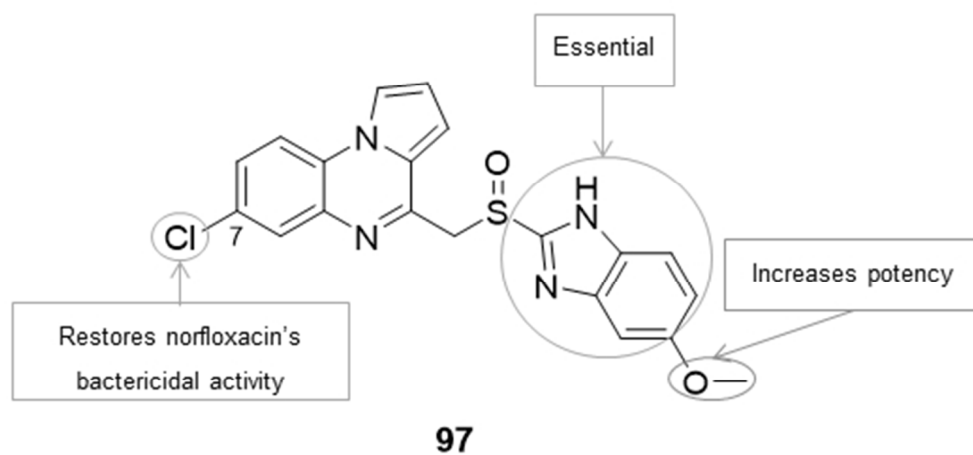


Figure 10 Conclusions from SAR studies for omeprazole derivatives.

141x69mm (96 x 96 DPI)

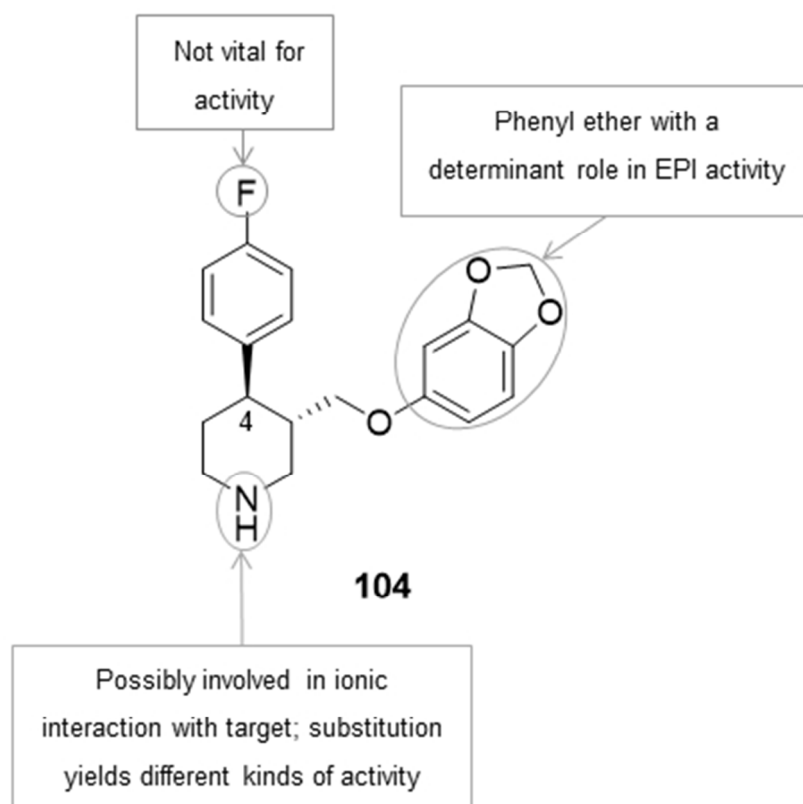


Figure 11 Conclusions from SAR studies for paroxetine derivatives.

109x111mm (96 x 96 DPI)

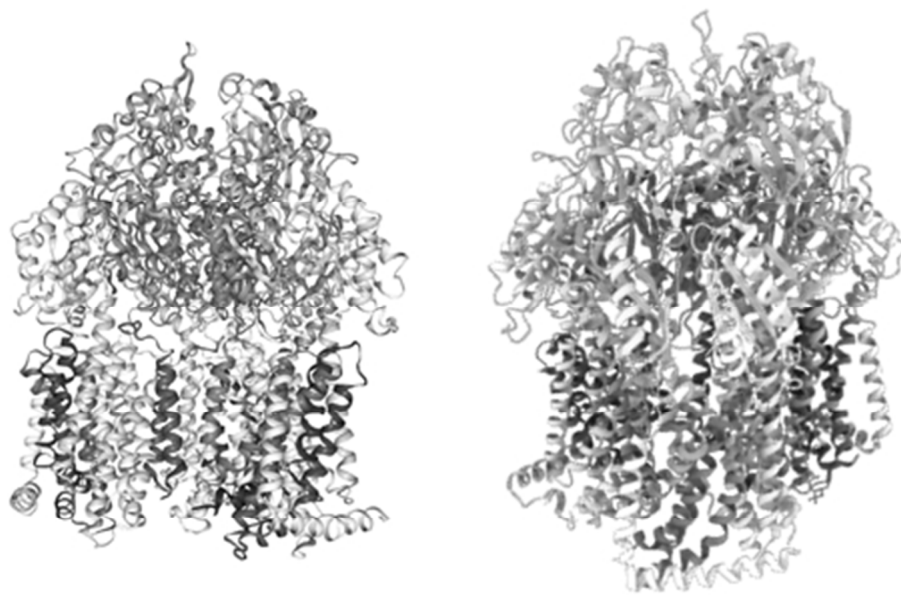
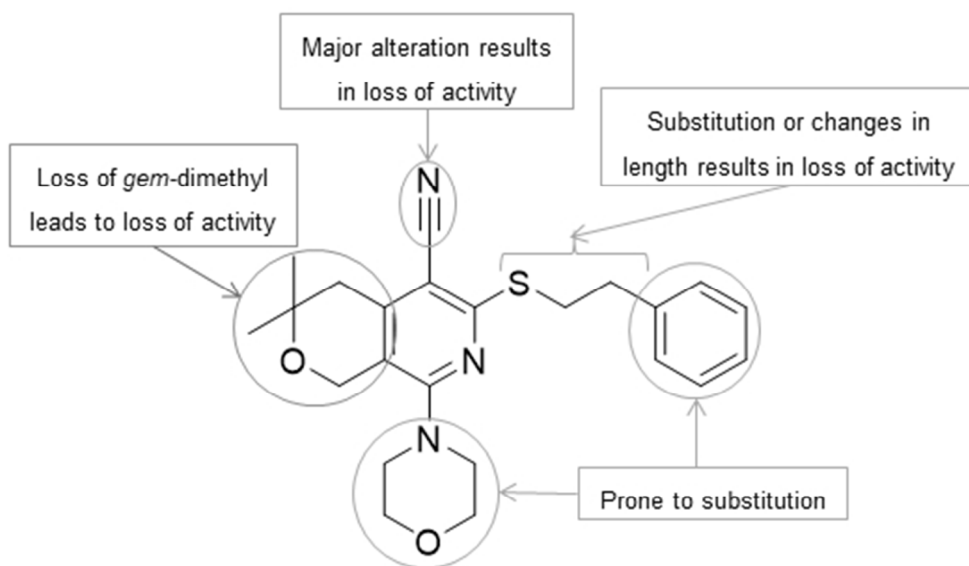


Figure 12 Crystal structures of AcrB (left, PDB 4ZLJ) of *E. coli* and MexB (right, PDB 2V50) of *P. aeruginosa*.

128x83mm (96 x 96 DPI)



28 Figure 14 Conclusions from SAR studies for MBX2319 (127) (adapted from [28]).

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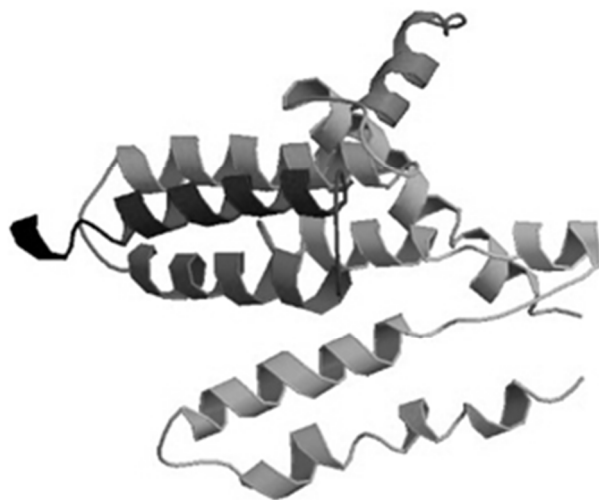


Figure 16 Crystal structure of EmrE (PDB 3B61) of *E. coli*.

83x67mm (96 x 96 DPI)

Review Only

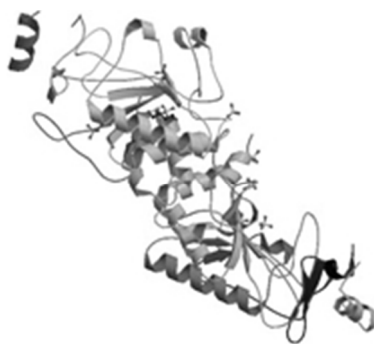


Figure 18 Crystal structure of MepA (PDB 1TZP) of E. coli in its inactive form.

53x45mm (96 x 96 DPI)

Review Only