Review

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Future Medicinal Chemistry

Membrane-targeting antibiotics: recent developments outside the peptide space

Catarina Dias[1,2] & Amélia P Rauter*[1,2]

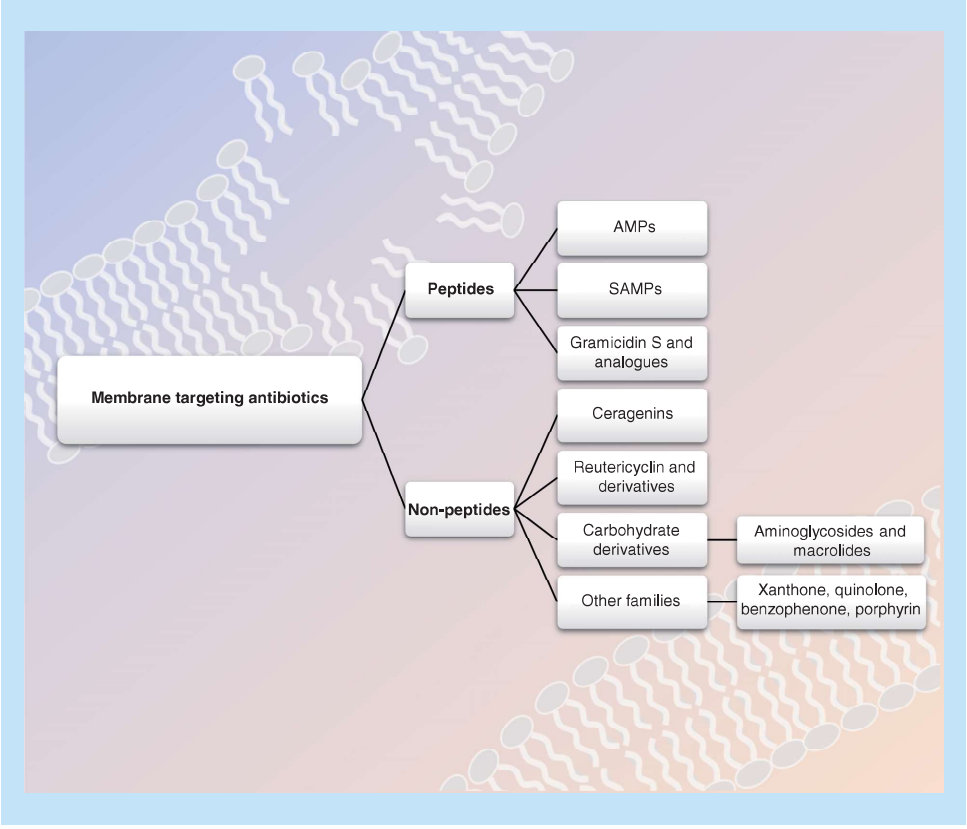
[1]Centro de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal

[2]Centro de Química Estrutural, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal

*Author for correspondence: Tel.: +351 217 500 075, aprauter@fc.ul.pt

The rise of antibiotic resistant bacteria requires unconventional strategies toward efficient chemotherapeutic agents, preferably with alternative mechanisms of action. The bacterial cell membrane has become an appealing target since its essential and highly conservative structure are key challenges to resistance mechanisms. Inspired by natural antimicrobial peptides, research on membrane-targeting antimicrobials has been growing out of the peptide space. The pursuit of more druggable molecules led to the discovery that the pharmacophore of antimicrobial peptides is smaller than anticipated. Several promising classes of membrane-targeting antimicrobials have been discovered, such as ceragenins, reutericyclines, carbohydrate amphiphiles – among others. This review will discuss the most recent findings on membrane-targeting antibiotics, focusing on small molecules outside the antimicrobial peptide molecular space.

Graphical abstract:



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The inadequate use of antibiotics over the past decades, allied to natural resilience mechanisms of bacteria, led to the rise of antimicrobial resistant strains to a point where antibiotic resistance is becoming a major society challenge of the 21st century. Antibiotics used currently in the clinic target biosynthetic processes, such as protein biosynthesis, RNA, DNA, peptidoglycan and folic acid, which lack efficiency against infections caused by quiescent bacteria [1]. These phenomena require unconventional strategies, making research on antimicrobial chemotherapy with alternative mechanisms of action of extreme importance.

Antimicrobial peptides (AMPs) are natural antibiotics that have been part of the innate immune system of microorganisms, and of humans, for hundreds of millions of years as a host defense mechanism [2,3]. Isolated for the first time in the late 1930s, this class of antibiotics acts mainly by interacting with negatively charged cell membrane with consequent loss of membrane integrity and presents surprisingly little resistance. A number of interaction models have been proposed to explain their mechanism of action, such as carpet-like, membrane thinning, toroidal pores and barrel-stove [4]. Moreover, recent studies on AMPs' mode of action revealed that they do not only affect membrane permeabilization, and some of them are also intracellularly active, inhibiting proteases, DNA and protein synthesis [2,4]. Nevertheless, these amazing natural peptides, which activity and mechanism of action have been extensively reviewed [4,5], are known for their toxicity as membrane disruptors, susceptibility to proteases and extreme pH, folding issues for some large AMPs and high production costs [4]. Aiming to circumvent these drawbacks, the scientific community has been encouraged to investigate smaller and more druggable molecules also able to modulate bacterial membranes even though the collective knowledge on the optimization of this chemical space is scarce. This review will display the most recent findings on membrane-targeting antibiotics, focusing on small molecules outside the antimicrobial peptide molecular space.

Lipid diversity: the key toward selectivity

Throughout all living organisms, a small hydrophobic layer defines the borders between a cell's life and death. Nevertheless, despite the well-known importance of lipids and their role in several essential functions, with a diversity similar to that of proteins, they are far from being as studied as the latter as drug targets [6]. However, regarding antibiotics, there is one aspect that makes targeting bacteria cell membrane so appealing: its essentiality. The bacterial membrane is essential independently of the metabolic status of the cell, as it is not only vital for homeostasis and metabolic energy transduction, but it also houses a third of cell proteins, which regulate a number of crucial roles. Thus, cell envelope ultrastructures cannot easily change without substantial loss of function, and the development of resistance becomes incredibly difficult, as observed for AMPs [1].

The major structural lipid in eukaryotic membranes is phosphatidylcholine (PC), accounting for more than 50% of all glycerophospholipids. Due to its geometry, it organizes spontaneously in a planar bilayer (Figure 1). Other phospholipids are also present, such as phosphatidylethanolamine (PE), phosphatidylserine, phosphatidylinositol and phosphatidic acid (Figure 1). The inclusion of the conical shaped PE in bilayers adds curvature, which is crucial to a number of roles, such as budding, fission and fusion [7]. These phospholipids gather almost exclusively in the inner surface of the bilayer, constituting about 30% of the cell membrane [8]. Conversely, prokaryotic cells have a rather different membrane composition, being mostly composed of PE and phosphatidylglycerol (PG) and devoid of PC. While Gram-negative bacteria can have up to 80% in PE (e.g., *Escherichia coli*), Gram-positive bacteria present diverse membranes with various proportions of PE, PG and cardiolipin (Figure 1) [9]. It is easily observed that mammalian and bacterial membrane phospholipids diverge in nature and relative proportion, and consequently, in biophysical and physical-chemical properties. For instance, while PC and PE are zwitterionic, PG and cardiolipin are anionic, resulting in bacterial membranes more negatively charged than their mammalian counterparts.

In water, lipids can aggregate in a variety of crystalline structures. The most commonly adopted structures by single lipids in aqueous buffer are the lamellar phase (lipid bilayer) and the hexagonal phase (H_{II}) (Figure 2) [10]. Lipid polymorphism is strongly dependent on the molecular shape of lipids while cone-like molecules (e.g., phosphatidylethanolamine) tend to pack into structures with high radii of curvature, cylindrical molecules (e.g., PC) organize into flat bilayers [10,11]. Although in biological membranes lipids adopt a bilayer structure, nonlamellar structures, such as inverted hexagonal phase play an important role in membrane processes, specifically membrane fusion, vesicle stability, transbilayer transport and pore formation [12,13]. Packing, mobility and lateral organization

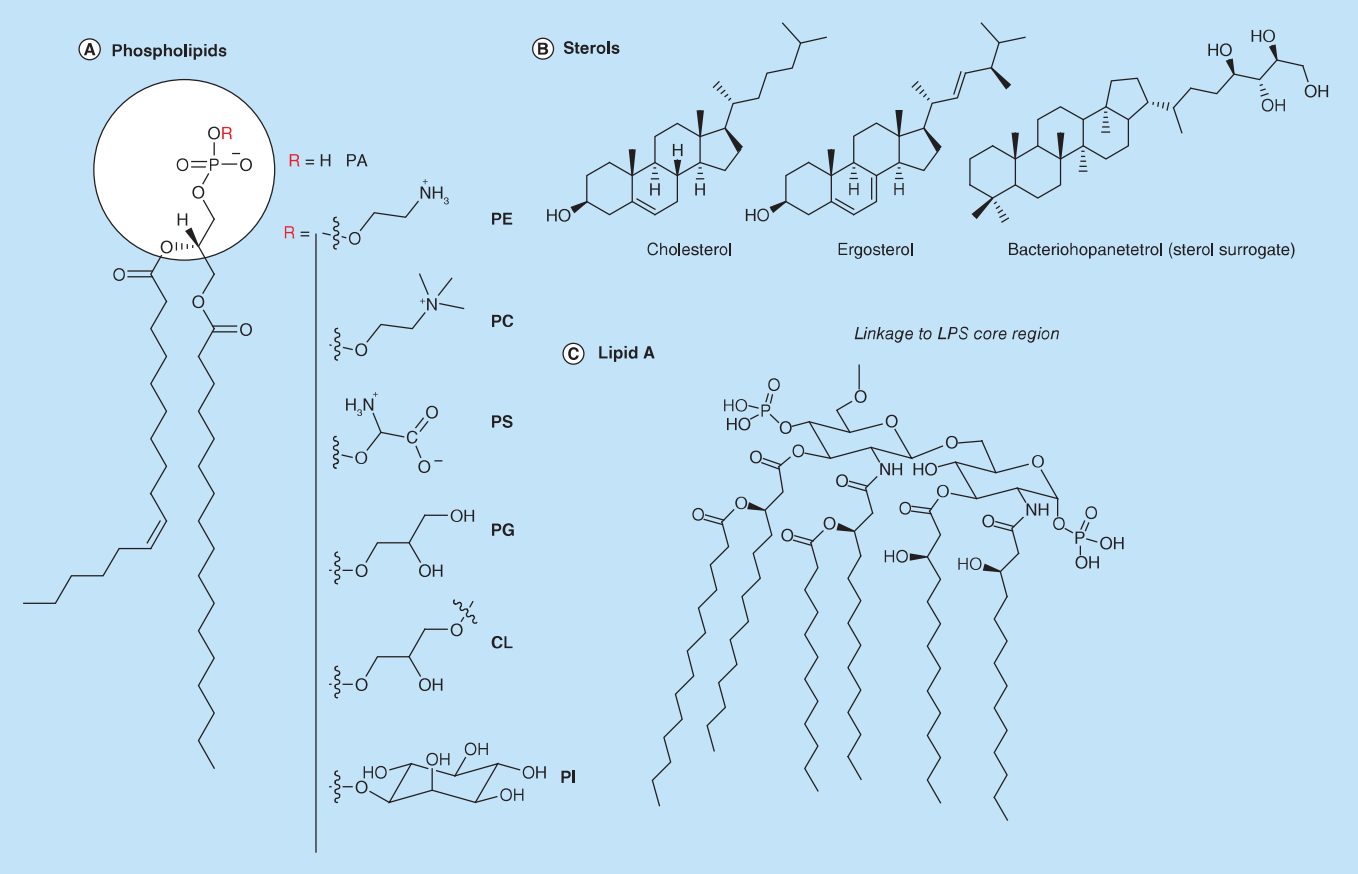

Figure 1. Membrane constituents. (A) Structures of the phospholipids PA, PE, PC, PS, PG, CL and PI. Acyl chain length and saturation positions are depicted arbitrarily. Acyl chain compositions and phospholipid relative amount vary among species, but the most common fatty acids to esterify glycerol-3-phosphate are palmitic, palmitoleic, steric and oleic acid. **(B)** Structure of cholesterol (mammals), ergosterol (fungi and plants) and bacteriohopanetetrol (some bacteria). **(C)** Structure of Lipid A of *Escherichia coli*. Lipid A constitutes the innermost region of lipopolysaccharide, that is attached to the outer membrane.
CL: Cardiolipin; LPS: Lipopolysaccharide; PA: Phosphatidic acid; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PG: Phosphatidylglycerol; PI: Phosphatidylinositol; PS: Phosphatidylserine.

of membranes are mainly dictated by the molar ratio of PG and PE, while other components (or membrane active molecules) can amplify or repress these properties [14].

This lipid diversity has the potential to be explored toward selectivity, where differential interaction of amphiphilic drug and bacterial cell versus host cell can play a key role. Indeed, a recent *in silico* study shows that the entry of an amphiphilic antimicrobial lipopeptide is more favorable in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPE:POPG) model membrane than in a membrane composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, enhancing the thermodynamic and kinetic selectivity for bacterial versus mammalian model membranes [15]. Moreover, an elegant study where AMPs maculatin and aurein were preincubated in a neutral lipid environment, and then introduced to charged lipid vesicles, showed that AMPs can be so selective that they translocate to anionic membranes [16].

Lipid headgroup and hydrocarbon chain length are known to strongly influence the mechanism of disruption by which membrane-active peptides act [14]. For instance, peptide-induced membrane defects are facilitated by the presence of asymmetric phospholipids containing iso-branched fatty acids, versus their anteiso-branched isomers, that melt at lower temperature [17]. In addition the binding of an antimicrobial peptide G4 to membrane lipids was shown to be dependent on the acyl chain saturation, being more prone to incorporate 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG) monolayers than POPG monolayers [18]. Better models of cell membranes would help, however, to understand why AMPs discriminate eukaryotic from prokaryotic cells.

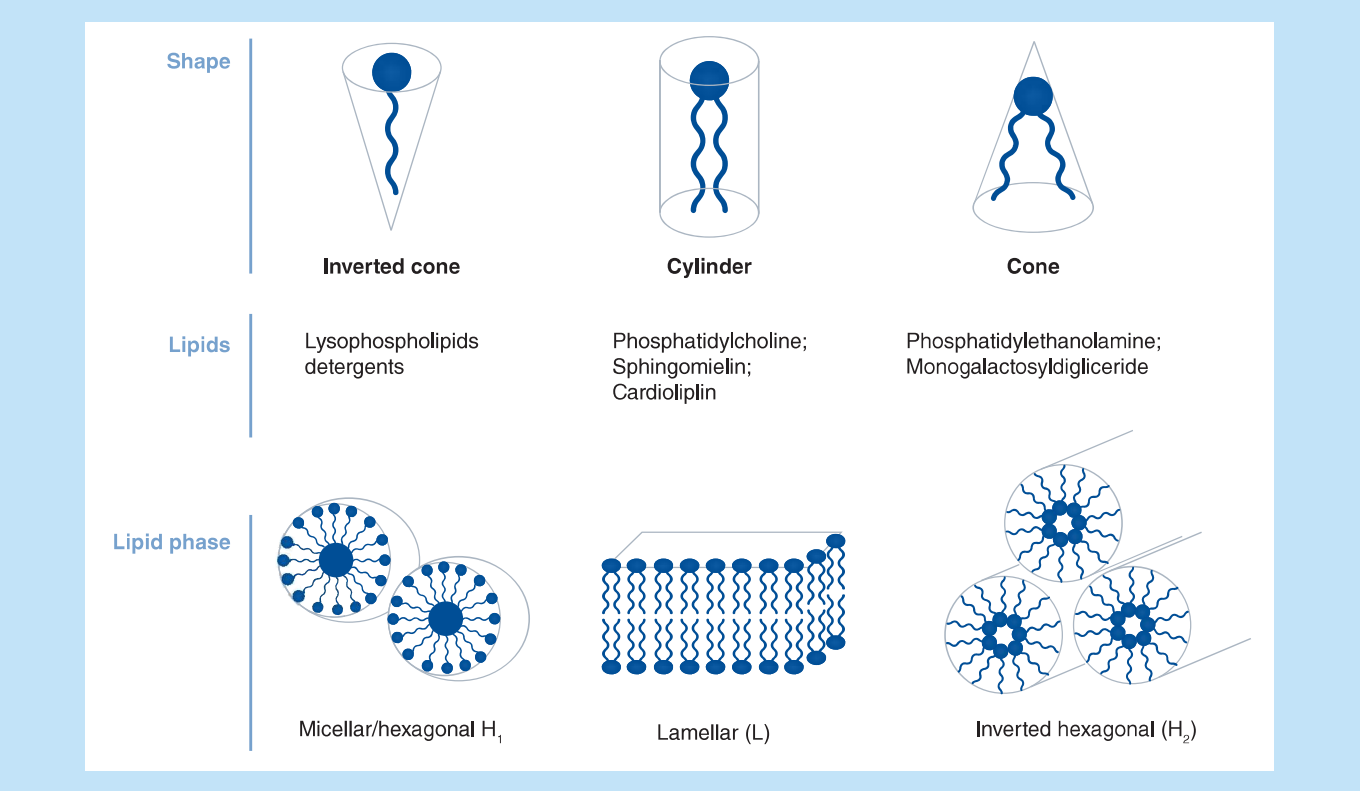

Figure 2. Lipid shape and polymorphism.

Not only phospholipids, but also sterols can modulate selectivity. Sterols' role is to maintain membrane integrity, by creating a microfluidity state in the membrane, which is essential for cell function on a large temperature scale. A specific sterol is the major component of a cellular membrane type. Mammalian membranes contain cholesterol while sito-/stigmasterol is present in plants and ergosterol in fungi (Figure 1).

Bacteria are generally devoid of sterols, with the exception of some primitive bacteria (archebacteria, cyanobacteria, etc.) that contain sterol surrogates such as the hopanoid bacteriohopanetetrol (Figure 1) [19]. While bacteria are susceptible to the action of AMPs, sterols seem to protect negatively charged membranes against peptide disruptive effects [20,21].

Interestingly, a comparative study showed that cholesterol is much more effective than ergosterol in preventing the intercalation of human host defense peptide LL-37 in membranes suggesting that sterol structure may have evolved as a mean to improve the resistance to natural AMPs, thus promoting their survival without jeopardizing AMPs' efficiency [22]. Still, the exact role of sterols on the toxicity and selectivity of AMPs is not fully understood. Further studies on the origin of the reduced affinity of many AMPs for cholesterol containing membranes could benefit the design of more efficient membrane-targeting antibiotics.

Peptides & analogs

Antimicrobial peptides

Antibacterial peptides were the first membrane-targeting antibiotics to be approved by US FDA. Most AMPs contain a net excess of positively charged residues and a size ranging from 12 to 50 amino acid residues, of which approximately 50% are hydrophobic in nature [23]. The potency of these peptides is generally in the micromolar range, which is characteristic of nonspecific mode of action. As already discussed, AMPs' mechanism of action relies mainly upon interaction with the bacterial membrane, although other phenomena have been identified, such as interaction with DNA as well as the release of natural defense peptides and the stimulation of phagocytic cells due to the innate immunity system [24]. The AMPs commonly assume an amphiphilic secondary structure where cationic amino acid moieties of residues, such as arginine, lysine and histidine, are oriented on one face of the molecule, while hydrophobic moieties are on the opposite side, resulting in the so-called facial amphiphilicity [25].

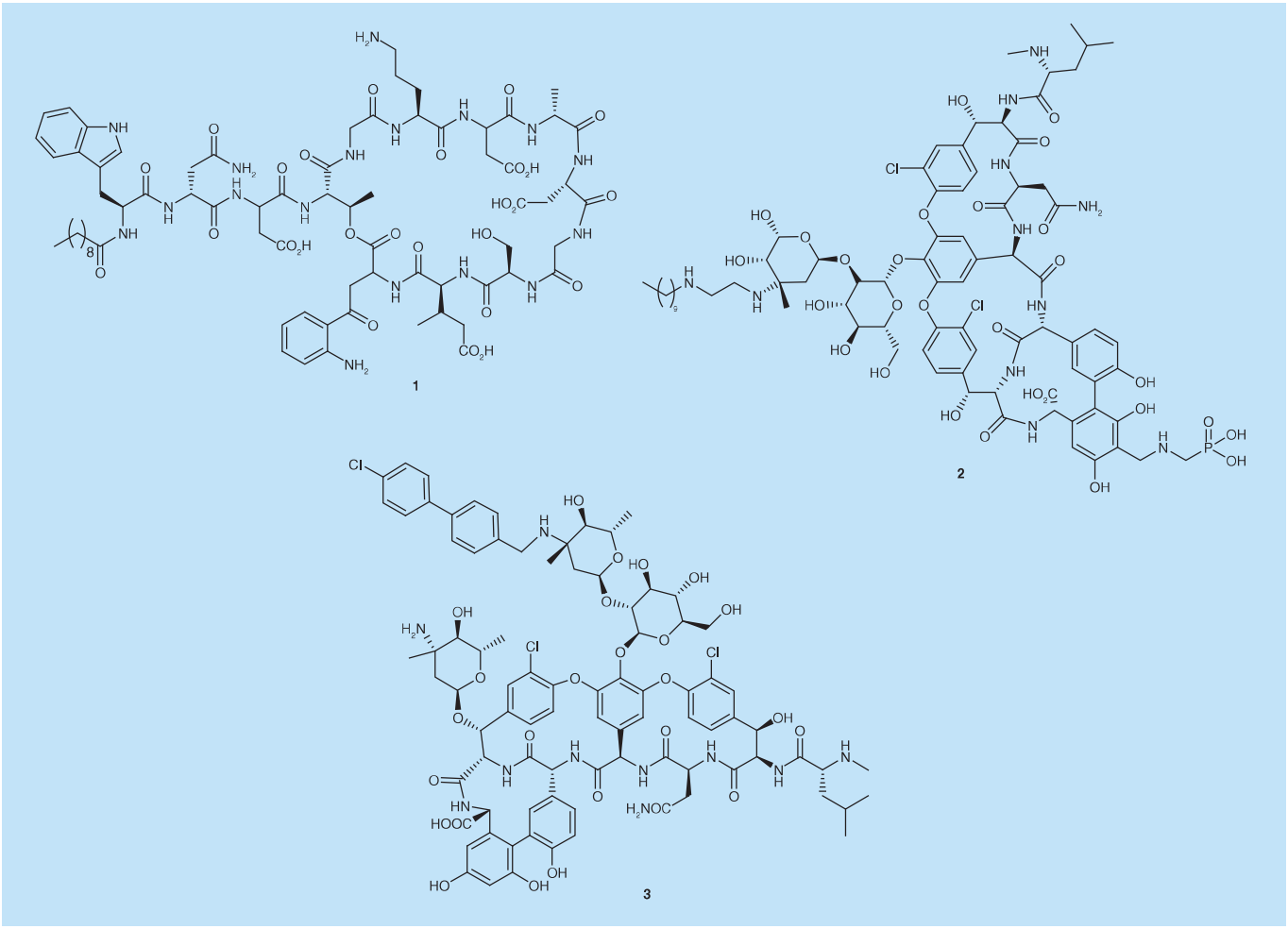

Figure 3. Antimicrobial peptides approved by the US FDA. Structures of **(1)** daptomycin, **(2)** televacin and **(3)** oritavancin.

They bind preferentially to negatively charged and zwitterionic phospholipids, exposed in bacterial membranes, giving them the necessary selectivity.

The main limitation of AMPs is their toxicity, guilty of many serious adverse effects, as well as their low *in vivo* stability due to high serum binding, absence of antimicrobial activity in physiological concentration of salts and protease degradation. For these reasons, the discovery of noncytotoxic AMPs requires a challenging compound optimization process toward appropriate physicochemical properties [26,27]. Furthermore, their complex design results in high production costs. However, the positive remarks on AMPs, such as lack of resistance, made them reach the market. The lipopeptides, daptomycin and televacin, (Figure 3) were the first antibiotics targeting the membrane approved by the FDA that are clinically used.

In 2014, the semisynthetic glycopeptide oritavancin (**3**; Figure 3), similar to the cell wall synthesis inhibitor vancomycin, was approved by the FDA for the treatment of skin infections. Oritavancin, acting as a peptidoglycan biosynthesis inhibitor, is active over *Staphylococcus aureus* resistant to methicillin and vancomycin, being also active over other resistant Gram-positive bacteria. However, its rapid bactericidal activity against biofilm mediated infections has been attributed to its ability to depolarize and permeate bacterial membranes [28]. Indeed, vancomycin is inactive against staphylococcal biofilms.

Despite their clear importance in the fight against resistant Gram-positive infections, peptides **1–3** have to be administrated intravenously. These peptides have inspired new avenues of investigation, which have been pursued in order to find more selective and smaller molecules, with the same antibacterial potential. Some of these lines of investigation will be disclosed in the fore coming sections.

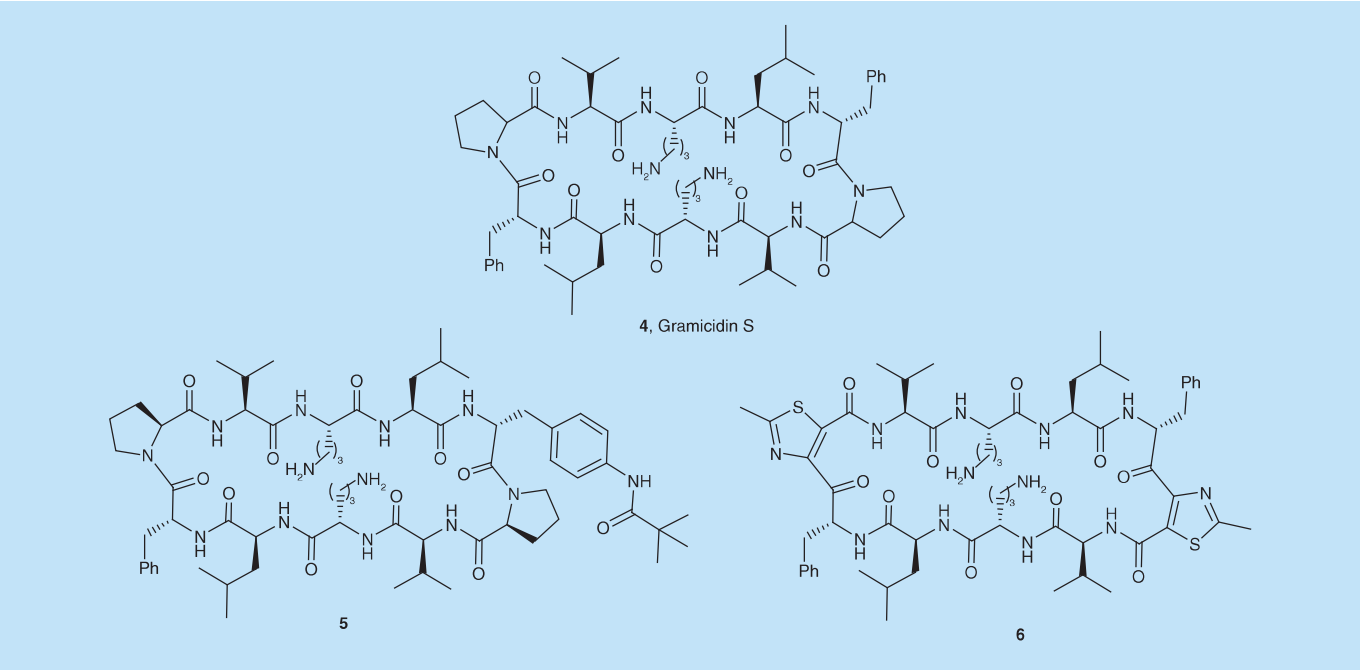

Figure 4. Structure of Gramicidin S and analogs.

Gramicidin S & synthetic analogs

Gramicidin S (GS, **4**; Figure 4) is a cationic cyclic decapeptide secreted by *Bacillus brevis*, discovered in the early 1940s, highly potent over pathogenic fungi, Gram-positive and Gram-negative bacteria [29,30]. This antibiotic exhibits a strong hemolytic activity, restricting its use to topical applications, although it saved many lives in the battle fronts of World War II where it was used to treat infected wounds [31]. Only later it was discovered that this antibiotic compromises the phospholipid bilayer barrier via a wide variety of transient, differently sized defects [32]. Some attempts to synthesize less toxic gramicidin S analogs have been described, including those bearing an aryl protected sugar mimicking one of the two proline residues (**5**; Figure 4), but this approach was not successful [33]. Later, the synthesis of asymmetric gramicidin S analogs, where the D-phenylalanine residue was modified to contain an exposed amide, provided one analog with a comparable antimicrobial activity as GS and a slightly reduced hemolytic activity [34]. More recently, Legrand and co-workers presented the synthesis of a new analog in which the D-phenylalanine-proline turn was replaced by a γ-amino acid with a thiazole ring (**6**; Figure 4). This derivative was able to maintain an antimicrobial activity very close to that of gramicidin S, while leading to a remarkably lower hemolysis, reducing it from 80 to 14% [35]. Transition of these analogs into clinic is yet to be reported.

Synthetic antimicrobial peptidomimetics

One approach to overcome AMPs limitations was to develop smaller antibacterial peptides. The preparation of a series of small peptides (2–5 amino acids) and peptide esters with the key features of an AMP – cationic charge and bulky hydrophobic residues – allowed us to understand that the pharmacophore was unpredictably small. For antistaphylococcal activity, a net charge of +2 and the presence of at least two bulky/lipophilic moieties are the only structural key features required. For instance, compound **7** (Figure 5) is active against *S. aureus*, methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. epidermidis* (MRSE) at 15, 15 and 10 μg/ml, respectively [23]. The resulting AMPs, later called synthetic antimicrobial peptidomimetics mimic the antibacterial properties of AMPs, while having improved pharmacokinetic properties [36]. More recently, a large tert-butyl substituted tryptophan was also synthesized. The Ltx5 (**8**, Figure 5), permeabilizes both the membrane of *E. coli*, and quickly eradicates *S. aureus* while keeping a high *in vitro* stability in human blood plasma, as well as low toxicity [36]. Later, it was shown that Ltx5, as well as other less active analogs, at a concentration ten-times higher than the MIC value, could eliminate *S. epidermidis* and *S. haemolyticus* biofilms metabolic activities [37].

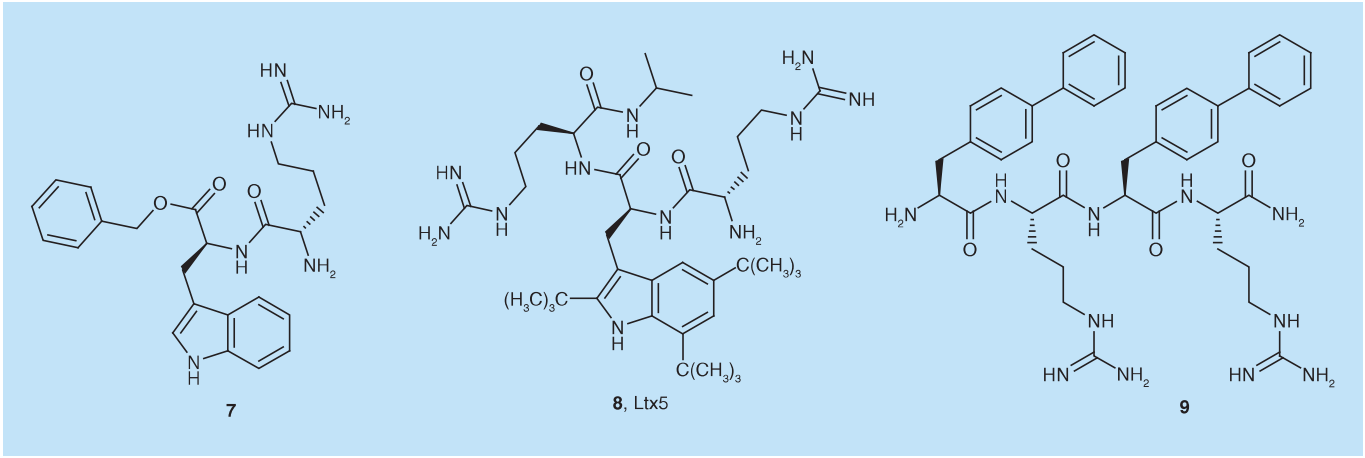

Figure 5. Structures of synthetic antimicrobial peptidomimetics embodying bulky aromatic moieties.

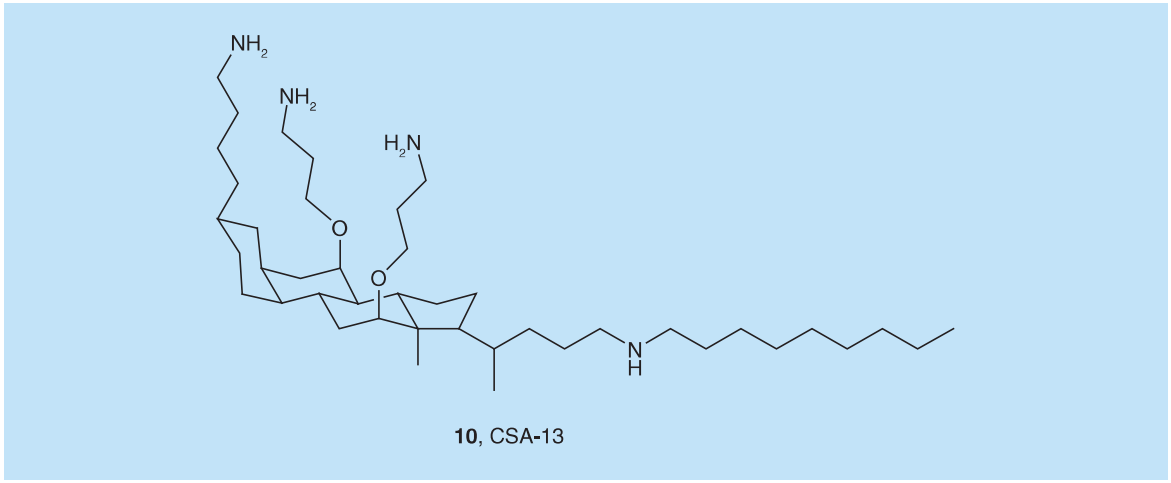

Figure 6. Structure of ceragenin CSA-13.

In 2018, a tetrapeptide **9** (Figure 5) comprising 4-l-biphenylalanin units, with a MIC of 6.25 μM against MRSA, was reported to act by negatively charged membrane disruption [38,39]. Upon insertion of the peptide in lipid-water interface, the line tension of the bilayer is disturbed causing lamellar-to-micellar phase transition, ultimately leading to lysis [39]. The low registered hemolytic activity (<1% at 100 μM) can be explained by peptide selectivity toward anionic membranes versus neutral ones [38,39], further supporting the possibilities in this chemical space.

Outside the peptide molecular space

Ceragenins

Ceragenins have a highly functionalized steroid core structure and mimic the activity of endogenous AMPs [9,25]. These steroidal compounds effectively reproduce AMPs facial amphiphilicity while being resistant to proteolysis and more suitable to large-scale synthesis. Many of these structures are polycationic and possess a lipophilic moiety, thus being capable of partitioning into membranes [9]. Their overall positive charge allows them to have selectivity against microbes with exposed anionic and zwitterionic lipids. Ceragenins are, in general, more potent over Gram-positive microbes but some of them are effective against both Gram-negative and Gram-positive bacteria. The most potent ceragenin to date is CSA-13 (**10**, Figure 6), considered a broad-spectrum antimicrobial being mostly active against Gram positive bacteria, such as *S. aureus* (0.4 μg/ml), *Streptococcus pyogenes* (0.5 μg/ml), *Bacillus subtilis* (0.5 μg/ml) and *Bacillus anthracis* (2.5 μg/ml), but also considerably active on the Gram-negative *E. coli* (3 μg/ml) and *Pseudomonas aeruginosa* (2 μg/ml) [9]. The CSA-13 has also shown potent activity against bacterial

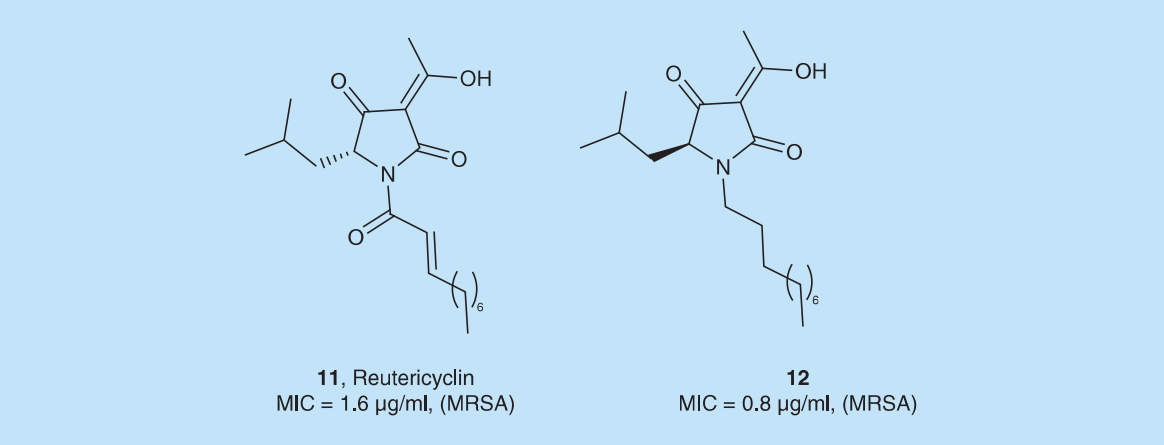

Figure 7. Reutericyclin and the structure of its analog, 12.
MIC: Minimal inhibitory concentration; MRSA: Methicillin-resistant *Staphylococcus aureus*.

biofilms [40]. The antimicrobial activity of ceragenins correlates well with a membrane composition containing a high concentration of phosphatidylethanolamine or uncharged lipids. It was demonstrated that ceragenins caused membrane depolarization, which is sufficient to cause lethality and can be seen as an indicator of potency [25]. While membrane depolarization is pointed as the primary mode of action of ceragenins, an intracellular target is yet to be excluded to influence antimicrobial activity. Remarkably, a recent study supports that *in vivo* activity of CSA-13 may additively and/or synergistically interact with host antibacterial molecules, helping the innate immune system to act on peritoneal *P. aeruginosa* infections [41]. The toxicity concern associated with amphiphilic molecules is also an issue with ceragenins. This was recently tackled by immobilizing CSA-13 on magnetic nanoparticles' surface, significantly decreasing the hemolytic activity while maintaining its strong antibacterial activity. This is possible due to an imine link between the ceragenin and the nanoparticle that hydrolyses in acidic environments, such as infection and inflammation sites [42]. In another approach, CSA-13 was combined with pluronic F-127 to explore the thermoreversible properties of this neutral surfactant for a drug delivery system. Despite the slight decrease in the bioactivity of this ceragenin while used with pluronic F-127, the significant inhibition of its hemolytic activity is encouraging. However, this study also pointed out that, as CSA-13's antibacterial activity is reduced in blood plasma, its application should be directed toward topical bacterial infections prevention or treatment [43].

Reutericyclin & derivatives

Reutericyclin (**11**, Figure 7) is produced by *Lactobacillus reuteri* isolates [44–46]. This tetramic acid antibiotic is active against Gram-positive bacteria, namely methicillin-resistant *S. aureus*, *Streptococcus* sp and *Clostridium difficile* [44]. It is a highly hydrophobic and negatively charged molecule, thus being able to partition into the cytoplasmic membrane although it does not form pores. Rather, it promotes proton translocation across the membrane and dissipates the transmembrane ΔpH, resembling the action of weak organic acid (acetic and sorbic acid) [45]. The structure of reutericyclin is still under development, particularly due to its chemically instability and consequent challenging synthesis. To overcome this, Hurdle and co-workers proposed a series of easily synthesizable reutericyclin derivatives, such as **12** (Figure 7), that were able to eradicate staphylococcal biofilms much more effectively than the original compound, unlike the standard topical antibiotic mupirocin, which is not able to eradicate biofilms [46]. Reutericyclines were then proposed to be developed as new topical antibiotics. However, attempts to further optimize the structure toward a more potent antibacterial activity also increased cytotoxicity in kidney cells, but the high selectivity index in some cases made toxic compounds some of them interesting for further development. The studies presented suggest that both activity and cytotoxicity are modulated by physicochemical properties of the molecule [44]. These development challenges are expected during membrane-targeting antibiotic molecules and are in line with the discovery of nontoxic AMPs, as discussed previously. Further investigation on reutericyclin derivatives is required before clinical studies.

Carbohydrate based amphiphiles

Aminoglycosides

Aminoglycosides are a group of antibiotics used as last resource treatment of serious systemic Gram-negative infections, that inhibit protein biosynthesis by binding to the 16s ribosomal RNA. Like other classic antibiotics, their widespread use led to the emergence of bacterial resistance, which, in association with the serious side effects they cause, contributed to their clinical decline [47]. In recent years, aminoglycosides have been revisited, and several groups have tried to incorporate hydrophobic units in these oligosaccharide structures to obtain membrane disrupting antimicrobial agents [48,49]. These so-called amphiphilic aminoglycosides (AAG) can result from introduction of one or several lipophilic groups on the AG amino and/or hydroxy groups (Figure 8). Parent aminoglycosides coupled with lipophilic moieties, include neomycin B (**13**), neamine (**14**), tobramycin (**15**) and paromycin. Simply by benzylating neomycin B hydroxy groups (compound **16**; Figure 7), antibiotic activity against Gram-positive bacteria was improved [50]. Schweizer and co-workers also reported that decoration of neomycin B with a palmitoyl moiety (compound **17**; Figure 8), followed by conversion of the free amines into guanidines resulted in a compound 32-times more potent than neomycin B against MRSA, with only 13% of hemolysis [51].

Successful alterations to neamine include its O-alkylation with naphtylpropyl groups (compound **18**; Figure 8). Such compounds presented an activity on Gram-positive bacteria of 2–16 μg/ml, and *in vitro* affinity to lipopolysaccharide (LPS) comparable with that of polymyxin B [52]. One of the most active compounds also showed a low toxicity in eukaryotic cells at 10 μM.

Fridman and his team have been trying to find the structural parameters essential to antimicrobial activity and membrane selectivity of tobramycin derived amphiphilic aminoglycosides [50,53]. They established that linear aliphatic chains are better than aryl based ones and, by altering the number, length, bond and position of said aliphatic chains, they were able to generate potent broad-spectrum AAG antibiotics, in some cases, 32-fold more potent than tobramycin. The most potent antimicrobials were the analogs embodying a C14 aliphatic chain, when compared with those with the corresponding C12 and C16 chain. Unfortunately, a linear correlation of tobramycin analogs' antimicrobial activity with their hemolytic activity could not be established as all parameters, including the type of linkage between hydrophobic and hydrophilic moieties affected both biological outcomes. However, amide bound C12 analog (compound **19**; Figure 8) was significantly more potent than tobramycin against a number of staphylococcal strains and caused little measurable hemolysis in rats. The analog AAG with the chain linked to tobramycin via a triazole was highly hemolytic. Some of the tobramycin derivatives, including compound **19**, also inhibited biofilm growth of *Streptococcus mutans* and *Staphylococcus epidermidis* (MBIC 4–64 μg/ml) [53]. Although these parameters only do apply to tobramycin, the optimized hydrophobicity/hydrophilicity ratio could be translated to paromycin derivatives. Coupling this aminoglycoside with two C7 aliphatic chains resulted in a potent antimicrobial activity (MIC 2–16 μg/ml) against several staphylococcal strains, including MRSA while presenting negligible hemolysis at 32 μg/ml [50].

The mode of action of these new aminoglycosides has not yet been disclosed, although its amphiphilic nature and their LPS affinity *in vitro* point to a membrane related mechanism. Nevertheless, it would be interesting to deeply understand that how compounds **16** to **19** interact with bacteria membranes.

Polyene macrolides

Polyene macrolides are potent antifungal agents currently applied in the treatment of superficial and invasive infections. These molecules, amphipatic in nature, are characterized by a large lactone ring with multiple insaturations, forming small diameter channels (ca. 0.6 nm) in the membrane of sensitive organisms, resulting in cell death. The most extensively studied polyene macrolides include amphotericin B (**20**), used in clinic in the treatment of invasive mycoses, and nystatin (**21**), its use is limited to topical infections due to its high toxicity despite its higher antifungal activity (Figure 9) [21,54].

The toxicity and severe side effects caused by polyene macrolides can be easily explained since they apparently target membranes containing sterols (ergosterol and cholesterol), common to both fungal and mammalian cells. Interestingly, a very recent work showed that the ability of nystatin to form and stabilize pores is not dependent on the presence of such sterols, but rather on the presence of highly ordered membrane domains [21]. In addition, the authors also showed that pore formation is accompanied by strong Nys-induced membrane reorganization, highlighting that, although ergosterol might be essential to Nys antifungal activity, other biophysical membrane properties need to be addressed to further understand the mechanism of action and toxicity of such molecules [21].

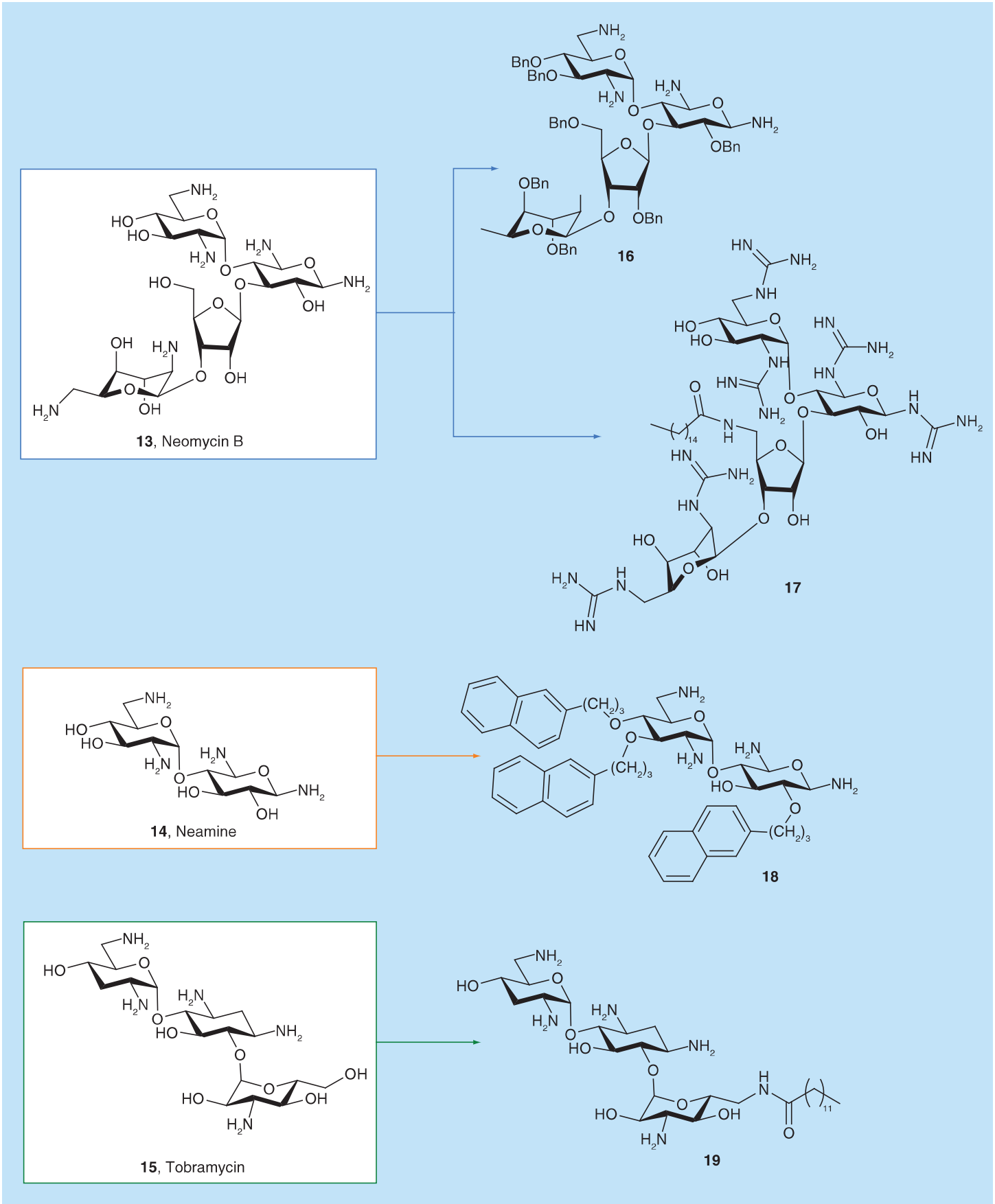

Figure 8. Structures of amphiphilic aminoglycosides 17–19 that were developed, starting from antibiotics (13) neomycin B (14) neamine and (15) tobramycin.

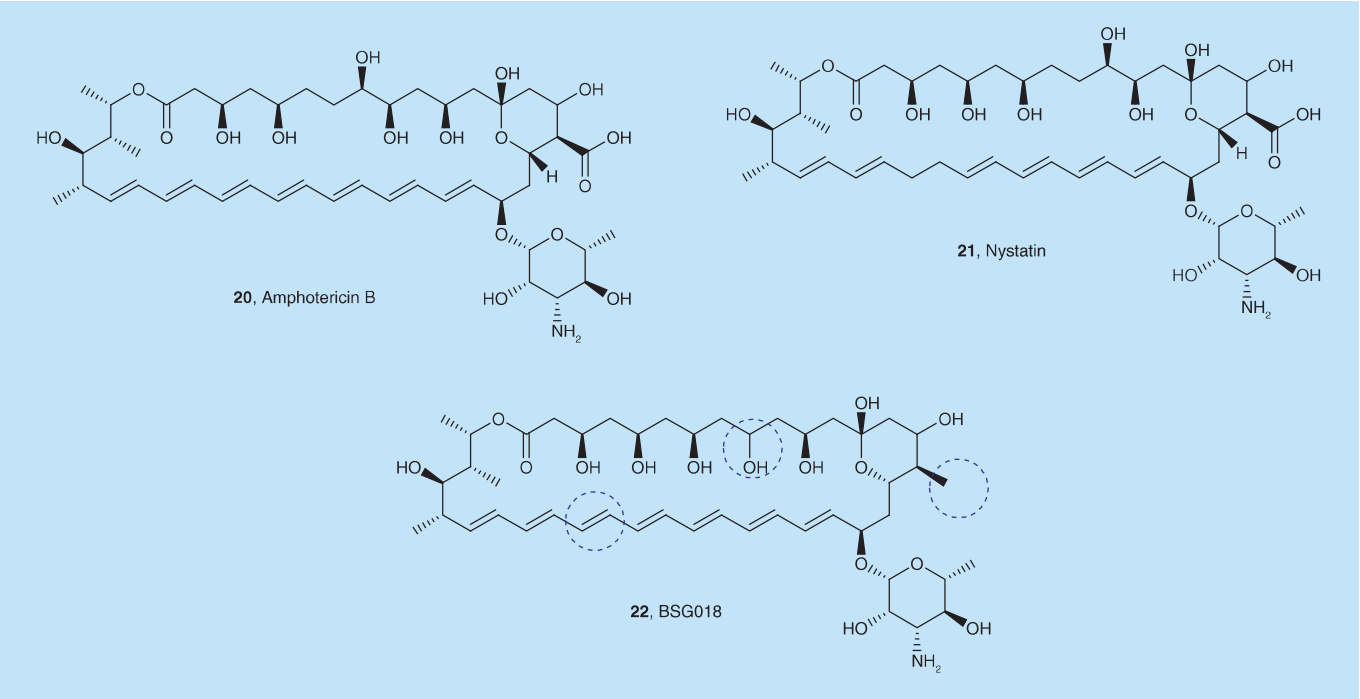

Figure 9. Polyene macrolides amphotericin B (20), nystatin (21) and BSG018 (22).

Alterations in the structure of polyene macrolides toward less toxic analogs have been attempted. Worth mentioning is the biosynthetic engineering of *Streptomyces noursei* that resulted in a series of nystatin analogs with changes the polyol moiety and the carboxyl group. Replacing the C-16 carboxyl with a methyl group and introducing a hydroxy group at C-9 (**22**; Figure 9) increased the antifungal activity and decreased the hemolytic activity, resulting also in twofold increased selectivity (relative to amphotericin B) [55].

Other structures

Xanthone derivatives

Recently, two xanthone derivatives symmetrically decorated with isoprenyl and arginine moieties (**23** and **24**; Figure 10), have shown potent antimicrobial activity against Gram-positive bacteria, in particular MRSA (0.78–3.13 μg/ml) and VRE (1.56–6.25 μg/ml) [56]. Given their amphiphilic and cationic character, their ability to disrupt bacterial membranes leading to the leakage of intracellular components comes with no surprise. These molecules are, however, surprisingly selective toward bacteria, causing 50% blood cell hemolysis (HC_{50}) only above 750 and 2000 μg/ml. This is allegedly due to electrostatic preference of negatively charged bacterial membranes over the zwitterionic eukaryotic cells. As already discussed, membrane-targeting antibiotics present little resistance since it is difficult for bacteria to remodel their membranes. The optimized xanthone derivatives were no exception, presenting no significant change in the MIC after bacterial exposure. In addition, compound **23** was successfully tested in an animal model of cornea infection [56].

Porphyrin derivative (XF73)

The dicationic porphyrin XF73 (**25**; Figure 10) is a fairly recent antistaphylococcal compound, developed by Destiny Pharma Ltd. With a MIC comparable wiht that of daptomycin, tetracycline and vancomycin (1 μg/ml), it acts by compromising the cytoplasmic membrane, inducing >90% reduction of membrane potential at 4x MIC, loss of intracellular cations and reduction of cell viability [57]. The XF-73 is currently in clinical development and successfully passed Phase I clinical trials [58].

Table 1. Summary of advantages and limitations of membrane-targeting antibiotics herein discussed.

Type	Antimicrobials	Mechanism of action	MIC (strains)	Hemolytic activity	Advantages	Limitations	Ref.
Peptides and analogs	AMPs (12–50 a.a.)	Membrane disruption (several models proposed)	0.1–10 μg/ml (broad spectrum)	HC_{50} = 2–200 μM	Low levels of induced resistance; broad-spectrum activity; rapid onset killing	IV administration; systemic and local toxicity Low *in vivo* stability; high production cost	[4,5,14]
	Gramicidin S and analogs (10 a.a.)	Membrane disruption	2–64 μg/ml (broad spectrum)	80 vs. 14% at 100 μM (GS vs. compound **6**) HC_{50} = 35.2 μM (GS)	Easier synthesis	Toxicity; susceptibility to proteases	[34,35]
	SAMPs (2–5 a.a.)	Membrane disruption	MIC = 1–20 μg/ml (broad spectrum)	HC_{50} = 705 μg/ml (Ltx5) Hem. <1% for X at 100 μM	Improved pharmacokinetics	Susceptibility to proteases	[23,38,65]
Non-peptides	Ceragenins	Membrane disruption through membrane depolarization	0.4–3.0 μg/ml (broad spectrum)	HC_{50} = 30 μg/ml (CSA-13) (Negligible at 100 μg/ml for MNP-CSA-13)	Broad spectrum; resistant to proteolysis active against biofilms	Toxicity (which may be overcome by drug delivery systems)	[25,42,43,66]
	Reutericyclin and derivatives	Membrane disruption by dissipating the transmembrane ΔpH	1.6–0.8 μg/ml (MRSA)	HC_{50} = 28–32 μg/ml	Active on biofilms	Chemical instability High toxicity, limited to topical application	[44–46]
	Amphiphilic aminoglycosides (AAG)	Not fully disclosed (LPS affinity *in vitro*)	2–16 μg/ml (several staphylococcal strains, including MRSA)	Negligible at 32 μg/ml	Biofilm growth ihnibition	Mechanism of action is not fully understood	[49–51]
	Polyene macrolides	Membrane disruption via formation of small channels	0.18 (**20**) and 9 μg/ml (**22**) (*Candida albicans*)	HC_{50} = 3.0 μg/ml (**20**) HC_{50} > 200 μg/ml (**22**)	Potent fungicidal activity, relatively low frequency of resistance	High toxicity (target sterol containing membranes) Limited to fungal infections	[54,55]
	Xanthone derivatives	Membrane disruption	0.78-6.25 μg/ml (MRSA and VRE)	HC_{50} > 750 μg/ml	Highly selective, little resistance	Limited to staphylococcal infections	[56]
	Porphyrin derivative (XF73)	Membrane disruption by membrane potential reduction	1 μg/ml (*Staphylococcus aureus)*	Not reported	Systemic safety and tolerability following nasal administration	Limited to staphylococcal infections; lacks development for alternative applications	[57,58]
	Quinolone derivative (HT61)	Membrane disruption by depolarization	4 and 8 μg/ml (MRSA, VISA and VRSA)	Not reported (Safe for topical application)	Can act as an enhancer of other antibiotics (e.g., chlorhexidine)	Limited to Gram-positive bacteria	[59–61]
	Benzophenone derivatives	Disruption of membrane potential	0.5–2 μg/ml (MRSA, VISA, VRSA and VRE)	No hemolytic activity up to 100x MIC	Highly selective toward bacteria membranes	Limited to Gram-positive bacteria	[63,64]

AMP: Antimicrobial peptidomimetic; GS: Gramicidin S; HC_{50}: Hazardous concentration at 50%; LPS: Lipopolysaccharide; MIC: Minimal inhibitory concentration; MRSA: Methicillin-resistant *Staphylococcus aureus*; SAMP: Synthetic antimicrobial peptidomimetic; VISA: Vancomycin-intermediate *Staphylococcus aureus;* VRE: vancomycin-resistant *enterococci;* VRSA: VanA-type *Staphylococcus aureus*.

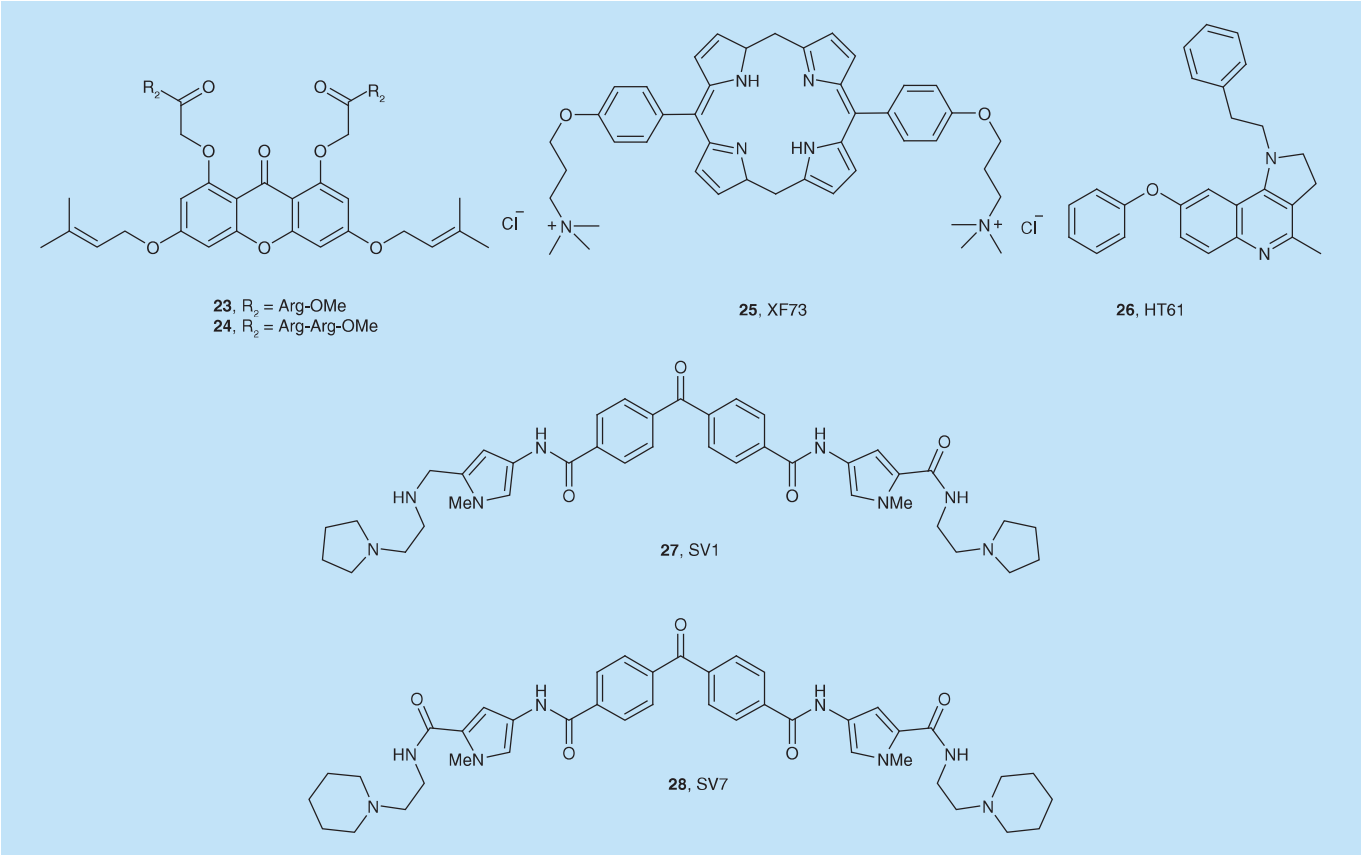

Figure 10. membrane-targeting antimicrobials containing the following aromatic moieties: xanthone (23, 24), porphyrin (25), quinolone (26) and benzophenone (27, 28).

Quinolone derivative (HT61)

The HT61 (**26**; Figure 10) is a quinoline-derived bactericide active against both quiescent and multiplying *S. aureus*. It also kills mupirocin resistant MRSA. The HT61 is able to kill bacteria in 2 h by depolarizing the cell membrane [59]. It was recently shown that, at concentrations both above and below the MIC, membrane depolarization and intercellular constituents' release take place, and that HT61 selectively binds to anionic lipids [60]. This quinolone derivative was also explored as enhancer of the antimicrobial activity of topical applications of neomycin, gentamicin, mupirocin and chlorhexidine, against both MSSA and MRSA [61]. Phase II clinical trial on the use of HT61 in anterior nares of subjects with nasal carriage of *S. aureus* was recently completed, but the results are not available [62].

Benzophenone derivatives

In 2009 a set of benzophenone-based antibiotics were presented, exhibiting MICs of 0.68–1.36 μM against the Gram-positive bacteria MRSA and VRSA (**27** and **28**; Figure 10) [63]. At the time, preliminary studies showed that **27** (SV1) and **28** (SV7) disrupted the membrane potential. Later, additional studies confirmed this mechanism of action and showed that this was a consequence of potassium ions release from the bacteria cell [64]. Interestingly, these compounds have also good affinity for lipoteichoic acids and other polyanionic components of Gram-positive cell wall membranes, as well as for LPS, which are part of Gram-negative membranes. These benzophenone-derived compounds do not show hemolytic activity at concentrations up to 100-times the MIC values.

Conclusion

The essentiality of cell membrane, adding to its highly conservative structure, makes it a promising target for new antibiotics and adds difficulty to drug resistance. The fact that bacterial membrane is essential independently of the metabolic status of the cell may be particularly relevant for persistent and biofilm mediated infections [1]. In addition,

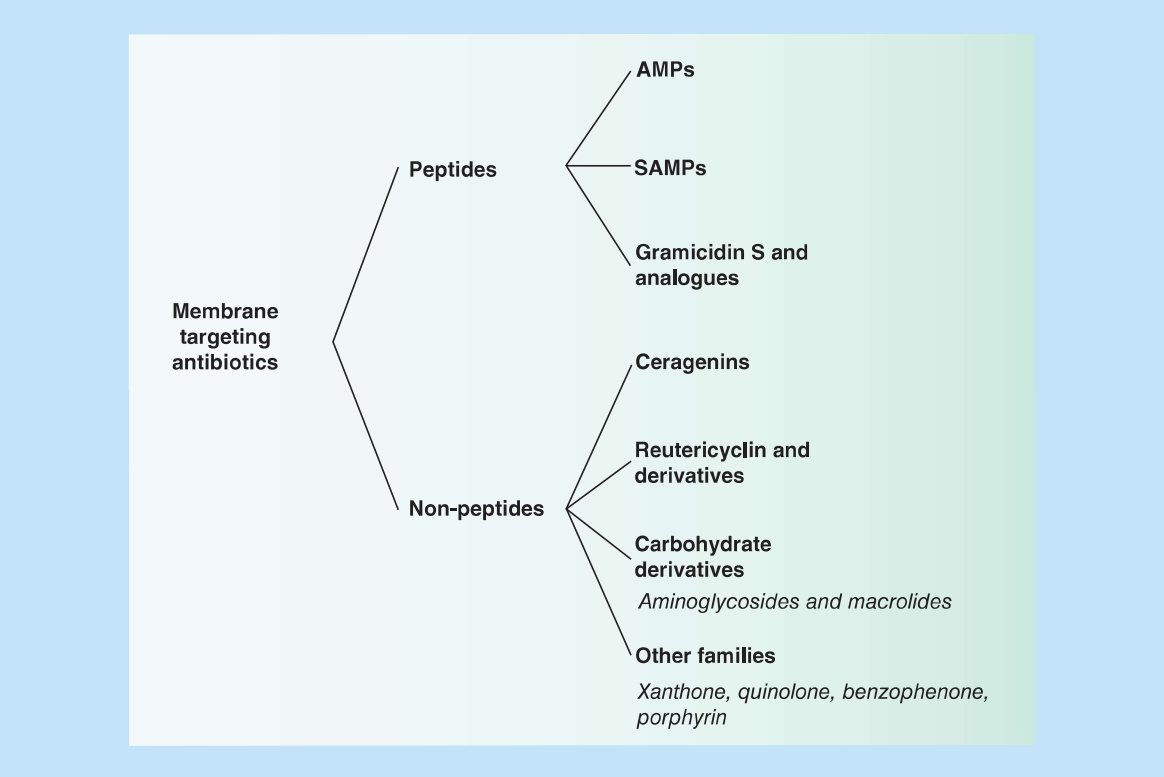

Figure 11. Structural diversity among membrane-targeting antimicrobials.
AMP: Antimicrobial peptidomimetic; SAMP: Synthetic antimicrobial peptidomimetic.

molecules-targeting cell membrane or cell wall can also be explored to act synergistically with conventional drugs, as they can compromise cell envelope permeability and integrity, increasing drugs' access and effectiveness [53].

However, this mechanism has been underexploited, and chemotypes with bacterial membrane disruption properties are often avoided and disregarded due to concerns over selectivity. In fact, most research on this field shows that the optimization toward membrane selectivity is highly variable and pattern identification has not been easy. The well-known differences between eukaryotic and prokaryotic cell membrane composition should be further explored, as demonstrated in this report. Most antibacterials here disclosed act by selectively binding to anionic lipids of bacteria or polyanionic components of the cell wall, such as lipoteichoic acid and lipopolysaccharides. The success of this strategy has been demonstrated for AMPs, the only membrane-targeting antibiotics to this date to reach the market. The pursue of more druggable molecules led to the discovery that the pharmacophore of AMPs is smaller than anticipated, which led to the disclosure of the so-called small AMPs. On the other hand, research outside the peptide space has been prolific, with several promising classes of membrane-targeting antimicrobials, such as ceragenins, reutericyclines, carbohydrate amphiphiles – among others. The repertoire here presented is structurally quite diverse and shown in Figure 11, while Table 1 summarizes antibiotic advantages and limitations, as well as the mode of action known and other relevant data known concerning their bioactivity and toxicity. Compound structures have in common the presence of an amine and a large lipophilic moiety(ies), which can be (but not limited to) an alkyl chain. Most of the compounds cited still need further optimization regarding membrane selectivity, but the results are promising, especially in an era where the fight against antibiotic resistance requires out of the box strategies.

Future perspective

Since the 1980s, new classes of anti-infectious agents are yet to be marketed, and resistance to the existing drugs continues to spread relentlessly. Despite the clear importance and essential functions of lipids and lipid ultrastructures, these have not been as well studied as proteins, in particular as drug targets. One characteristic of membrane phospholipids makes them particularly appealing targets for new antibiotics as cell envelope ultrastructures cannot

easily change without substantial loss of function and development of resistance becomes incredibly difficult. The membrane is indeed in a dynamic state with components crossing the membrane or forming polymorphic structures. Inhibition of such essential membrane processes can inspire new strategies toward antibiotic design. Moreover, antimicrobial therapy by targeting enzymes engaged in the biosynthesis of membrane components could also be further explored in long term.

This review has shown that lipid diversity has the potential to be explored toward selectivity, where differential interaction of amphiphilic drug and bacterial cell versus host cell can play a key role. However, it also showed that the structural optimization of the described molecules is very often challenging and time consuming. In fact, most of the compounds cited are charged and still need further optimization regarding membrane selectivity. The discovery of new, neutral and selective compounds is indeed encouraged, and this paper aims at inspiring research in this area. This is undoubtedly a rich and complex field that is just starting to be explored.

Executive summary

Lipid diversity: the key toward selectivity

- Nowadays, the fight against antibiotic resistance requires out of the box strategies.
- The essentiality and highly conservative structure of cell membrane makes it a promising target for new antibiotics and adds difficulty to drug resistance.
- Chemotypes with bacterial membrane disruption properties have been avoided and disregarded due to concerns over selectivity and challenging structure optimization.
- The differences between eukaryotic and prokaryotic cell membrane composition can be explored to attain membrane selectivity – while eukaryotic membranes major component is phosphatidylcholine lipids, prokaryotic cells are mostly composed of phosphatidylethanolamine and phosphatidylglycerol, and devoid of phosphatidylcholine.
- Most antibacterials that target bacterial membranes act by selectively binding to anionic lipids of bacteria or polyanionic components of the cell wall.

Peptides & analogs

- Antimicrobialpeptidomimetics (AMPs) were the first membrane-targeting antibiotics to be approved by US FDA. Examples include, daptomycin, televacin and oritavancin.
- The AMPs bind preferentially to negatively charged and zwitterionic phospholipids, exposed in bacterial membranes.
- Their limitations include low bioavalability and high toxicity, resulting in serious adverse effects.
- Smaller synthetic antimicrobial peptidomimetics containing 2–5 aminoacids have been developed, in which AMPs pharmacophore is significantly small.

Outside the peptide molecular space

- New avenues of investigation have been recently pursued in order to find more selective and more druggable molecules.
- Examples are structurally diverse and include cholic and tetramic acid derivatives, carbohydrates, xanthone, quinolone, benzophenone and porphyrin derivatives.
- Structural features shown include mostly an amine and a large lipophilic moiety(ies), which can be, but is not limited to, an alkyl chain.
- Most of the compounds cited still need further optimization regarding membrane selectivity, but the results are promising.

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