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MINI REVIEW

Structure–activity relationships of antibacterial peptides

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Abstract

Antimicrobial peptides play a crucial role in innate immunity, whose components are mainly peptide-based molecules with antibacterial properties. Indeed, the exploration of the immune system over the past 40 years has revealed a number of natural peptides playing a pivotal role in the defence mechanisms of vertebrates and invertebrates, including amphibians, insects, and mammalians. This review provides a discussion regarding the antibacterial mechanisms of peptide-based agents and their structure–activity relationships (SARs) with the aim of describing a topic that is not yet fully explored. Some growing evidence suggests that innate immunity should be strongly considered for the development of novel antibiotic peptide-based libraries. Also, due to the constantly rising concern of antibiotic resistance, the development of new antibiotic drugs is becoming a priority of global importance. Hence, the study and the understanding of defence phenomena occurring in the immune system may inspire the development of novel antibiotic compound libraries and set the stage to overcome drug-resistant pathogens. Here, we provide an overview of the importance of peptide-based antibacterial sources, focusing on accurately selected molecular structures, their SARs including recently introduced modifications, their latest biotechnology applications, and their potential against multi-drug resistant pathogens. Last, we provide cues to describe how antibacterial peptides show a better scope of action selectivity than several anti-infective agents, which are characterized by non-selective activities and non-targeted actions toward pathogens.

INTRODUCTION

The word "antibiotic", from the Greek ἀντι anti, "against" and βίος bios, "life", was coined by Selman A. Waksman in 1942, by announcing streptothricin (Waksman & Woodruff, 1942) to identify substances of microbial origin that acted against both Gram-positive and some Gram-negative bacteria. Such compounds inhibited the growth of various bacteria with bacteriostatic or bactericidal mechanisms. Antibiotics and antibacterials are both parts of the big family of anti-infective agents that also includes antifungals, antimycobacterials, antiprotozoals, and antivirals. Each class of anti-infectives can then be divided based on the specific action mechanism or on the chemical structure. Many applications such as cosmetics, imaging, biosensors, prosthetics, tissue engineering, and hard tissue engineering, make massive use of peptides, however here the authors aim at showing their potential as antibacterials.

Their discovery is simultaneous with the finding of innate immunity markers (Yuchen et al., 2020). Innate immunity mediated by peptides is different from adaptive immunity (based on lymphocytes and immunoglobulins; Zasloff, 2002), as it acts via a non-specific immune response with pro-inflammatory actors such as basophils, eosinophils, mast cells, natural killer cells, cytokines and other molecules with antimicrobial and antiviral activity.

Because of their heterogeneity, a standard classification of antibacterial peptides is rather difficult. Hence, we will describe them in relation to their chemical structure,

highlighting both how Mother Nature may still inspire the design of novel natural product analogues and how synthetic biology can play a key role in the difficult fight against multi-drug resistant pathogens (Ciulla & Kumar, 2018). A considerable number of peptide drugs are present in the antimicrobials family: due to their diversity and non-selective activity, they range from antibacterials to antifungals and antiviral peptides (Ballard et al., 2020), encompassing also anti-coronavirus peptides introduced during COVID-19 pandemic (Kalam et al., 2022). In this review we will focus on the antibacterial activity of peptides particularly suited for clinical applications in humans, displaying their importance in host defence, also considering the increasing need for novel antibacterial drugs effective against emerging drug-resistant microbial infections (de Barros et al., 2019). Indeed, since the increase of multi-drug resistance has become a global problem, alternative strategies for the design and production of innovative antibiotics are urgently required. To this purpose, natural antibacterial peptides could be a driving force in antibiotics discovery thanks to their biocompatibility, biodegradability, and minor toxicity compared to current commercially available small molecule antibiotics. Therefore, a deep knowledge of antibacterial structure–activity relationships (SARs) and a subsequent structural design strategy is highly necessary to build a library for high throughput screenings of molecules with high activity and selectivity against resistant strains. Literature reported that more than 3000 antimicrobial peptides (AMPs) were identified and characterized over the years (Wang et al., 2016). Interestingly, an updated version of the AMP database (APD, http://aps.unmc.edu/AP/) reported that 75% of AMPs derives from animals while a minority percentage originates from plants and bacteria, and only a very small part from archaea, protists, and fungi (Wang et al., 2016).

However, the therapeutic protein database referred that only 852 are therapeutic peptides, and 239 of those 852 were validated by Food and Drug Administration (FDA, www.fda.gov/drugsatfda). Out of 239 only seven are clinically approved AMPs: Gramicidin, Daptomycin, Colistin, Vancomycin, Oritavancin, Dalbavancin and Telavancin (Table 1). Recently, other more than 200 peptides are in preclinical evaluation (Henninot et al., 2017) suggesting that some advances are emerging to outcome some limitations in peptide drug discovery.

MOLECULAR STRUCTURE-BASED CLASSIFICATION OF ANTIBACTERIALS

Nature has manufactured and used a large number of AMPs to fight infections (Peacock et al., 2020): most of the time, they remained well-conserved during genome evolution (Mahlapuu et al., 2016). AMPs usually

TABLE 1 Seven FDA-approved AMPs, describing their mechanism of action, T1/2 (half-life), and important tips regarding their pharmacokinetics/pharmacodynamics (PK/PD).

FDA approved antimicrobial peptides	Mechanism of action	Half life	PK/PD considerations	Ref.
Colistin	Membrane lysis	3.5 h in healthy adults 48–72 h in patients with renal failure	Significant renal and neurologic toxicity	Mohamed et al. (2016), Sauberan and Bradley (2018)
Gramicidin	Membrane disruption and permeabilization by acting as a channel	Not yet available	It is highly hemolytic presenting restricts use to topical applications	Barboiu et al. (2014), Kelkar and Chattopadhyay (2007)
Daptomycin	Inhibition of bacterial growth by depolarization of membrane potential	7.5–9 h in healthy adults >28 h in patients with renal failure	Daptomycin tolerated if administrated once daily (dose 8 mg/kg for 14 days)	Carpenter and Chambers (2004), Chen and Lu (2020), Kreutzberger et al. (2017)
Vancomycin	Inhibition of cell wall synthesis	3–13 h in healthy adults 120–140 h in patients with renal failure	Because of the demonstrated dose-toxicity relationship of "red man" it has a narrow therapeutic window	Rybak (2006), Vandecasteele et al. (2013)
Oritavancin	Membrane lysis and Inhibition of cell wall synthesis	>10 days	As 85% of oritavancin is protein bounded, the active concentrations is significantly less	Rosenthal et al. (2018), Zhanel et al. (2010)
Dalbavancin	Inhibition of cell wall synthesis	>1 week (346 h)	Dalbavancin has dose-related pharmacokinetics	Chen et al. (2007), Smith et al. (2015), Zhanel et al. (2008)
Telavancin	Membrane lysis and Inhibition of cell wall synthesis	6.5 h	Multiple doses may lead to accumulation of telavancin in blood	Cavanaugh et al. (2019), Higgins et al. (2005)

feature low molecular weight, from 12 to 50 amino acids in length, a net positive charge (given by an excess of lysine and arginine over acidic residues; Hancock, 2001) and around 50% hydrophobic residues, that are primarily responsible for their antibiotic activity (Forde & Devocelle, 2015).

While more than 3000 natural-derived AMPs are listed in peptide databases, among them, just a handful are being tested in clinical trials (Eyler & Shvets, 2019; Wang et al., 2009). Indeed, only 30 new compounds entered clinical trials since 2000 (Hanna et al., 2021).

This is because the efficiency of antimicrobial treatments is also dramatically dependent on pharmacokinetics and pharmacodynamics (Yılmaz & Özcengiz, 2016). Pharmacokinetics and pharmacodynamics of AMPs (and their effects) are quite complex to investigate, but they have to be considered: the clinical development of antibacterial peptides is susceptible to undesirable pharmacodynamics due to peptides instability which leads to their degradation by proteolytic inactivation (Nguyen et al., 2010). Other important limitations to their clinical use include rapid clearance by kidney and liver (Vlieghe et al., 2010), short half-life in circulating plasma (Fosgerau & Hoffmann, 2015), and hemolytic activity (Pedron et al., 2019). For these reasons, new technologies and modifications should be considered to improve their bioavailability.

Peptide-based antibiotics exhibit diverse mechanisms of action, which include disruption of membrane integrity or interference with cell wall biosynthesis, protein synthesis, DNA replication, RNA transcription, and fatty acid biosynthesis (Nguyen et al., 2011; Nolan & Walsh, 2009; Velasco et al., 1997; Wang, 2014).

The principal mechanisms of action of peptide-based antibiotics are bactericidal (cell barriers disruption causing cell lysis; Madani et al., 2011) and bacteriostatic (nucleic acids binding and modulation of bacteria essential functions) effects (Jenssen et al., 2006).

Although bactericidal activity describes an immediate and definitive effect on bacteria's life, on the other hand, antimicrobials with bacteriostatic effect cause the stall of bacteria activity without directly causing death (Loree & Lappin, 2022). In non-peptidic antibiotics, the bacteriostatic activity usually comprises of inhibitory of bacterial protein synthesis pathway, (i.e., inhibition of bacterial ribosomes), such as in tetracycline antimicrobials that act as reversible inhibition of 30S ribosomal subunit arresting the bacterial protein production. AMPs mostly act with a bactericidal direct effect on bacteria targets. However, others play a role as bacteriostatic involving immune modulatory effects and enhancing the body's anti-infective response, such as inducing the synthesis of pro-inflammatory factors and secretion of cytokines (Lei et al., 2019).

The understanding of cell-penetrating peptides (CPP) may also be the key to transmembrane transport and drug delivery applications (Zorko & Langel, 2005).

AMPs are usually classified according to their predominant and activity-determinant secondary structures: α-helical, β-sheet, random-coil, or both α-helical and β-sheet structures (Lei et al., 2019; Takahashi et al., 2010).

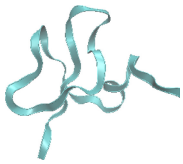
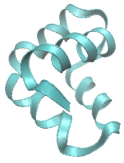
Due to their enormous diversity, antibacterial peptides can be hardly shown according to their source or mechanism of action, but the most adequate and not reductionist approach for their classification should consider their amino acids composition and secondary structure criteria. To this purpose, two major groups can be distinguished: linear and cyclic peptides. Linear peptides encompass (1) linear peptides with α-helical structures and no cysteines in their sequences; (2) linear peptides with predominantly β-sheet structures containing multiple disulfide bonds (Andreu & Rivas, 1998); (3) random coiled peptides rich in proline, arginine, tryptophan and/or histidine (Marimuthu et al., 2020). A large number of X-ray and 2D-NMR experiments (both in solution and in membrane mimetics) were reported to identify and determine the secondary and tertiary structures of such linear peptides (Ramamoorthy, 2009; Zhang et al., 1992). Ring formation can be achieved by covalent bonding, yielding to amides, ethers, lactones and thioethers (Choi & Joo, 2020). Cyclic peptides are present in nature, and ongoing efforts are focused on improving the protocols for their synthesis due to their advantageous properties such as, for example, their rigid structures. Indeed, rigidity is a fundamental asset of cyclic antibacterial peptides as it provides high metabolic stability (Góngora-Benítez et al., 2014), better membrane permeability (Kwon & Kodadek, 2007), and good oral bioavailability (Nielsen et al., 2017).

Linear peptides

The first studies on linear AMPs took inspiration from the immune system of insects, because of their ability to resist different bacteria with whom they are constantly in contact. Insects spontaneously produce an ensemble of peptides and proteins with antibacterial activities in response to their exposure to infectious microorganisms (Akuffo et al., 1998). Therefore, antibacterial proteins from insects are carefully studied and characterized, also because the insect immune response shares significant similarities with mammals.

Cecropins were the first antibacterial peptides from insects identified in the giant silk moth *Hyalophora cecropia* (Hultmark et al., 1980; Kimbrell, 1991). As a major player in the insect immune system, they show amphipathic helices and potent Gram-positive and Gram-negative antibacterial activity. The first mammalian cecropin was isolated by Boman from the pig intestine with a 64%–75% homology with *Hyalophora* and *Drosophila* (Lee et al., 1989). Cecropin P1 (Table 2) is active against several clinically relevant bacteria,

TABLE 2 Main classes of antibacterial peptides: examples, subclasses, and primary activity.

Class	Subclass	Examples	Net charge	Source	Secondary structure	Activity and selectivity
Linear	With α-helical structures and no cysteines	Cecropin P1	+4	Mammalian, porcine		GRAM-positive, Gram-negative bacteria
	Linear peptides with predominantly β-sheet structures containing multiple di-sulfide bonds	HBD-3	+11	Mammalian, human		Gram-positive bacteria MRSA MSSA
	Random coiled peptides rich in proline, arginine, tryptophan and/or histidine	LL-37	+6	Mammalian, human		Gram-positive, Gram-negative bacteria
Cyclic		Aureocin A53	+8	Bacteriocins		Gram-positive bacteria MRSA
Lipopeptides		Daptomycin	−3	*Streptomyces*		Gram-positive bacteria MRSA MSSA

Note: Molecular structures are from Protein Data Bank in Europe (https://www.ebi.ac.uk/pdbe). Net charges are described alongside and calculated for pH = 7.
Abbreviations: MRSA, Methicillin Resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *S. aureus*.

including *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Minimal inhibitory concentration (MIC) was determined to be 3 μM in vitro in *Escherichia coli* (Arcidiacono et al., 2009).

Attenuated total reflectance-Fourier transform infrared spectroscopy studies on mammalian cecropin P1 have revealed that the peptide does not penetrate in the membrane hydrophobic layer, but it generates a localized destabilization of the bacterial membrane, causing its alteration and disruption (Sitaram & Nagaraj, 1999).

Other insect antibacterial peptides and proteins are attacins, lysozymes, defensins, and proline-rich peptides: several members of these families share similitudes with antibacterial properties typical of mammals (Zhou et al., 1997).

Linear cationic amphipathic peptides were identified in amphibians: among them, magainin can be considered a major player. This class includes linear peptides from *Bombina* species, (bombinins, as BLP family; Simmaco et al., 1991), from *Phyllomedusa* genus (darmaseptins; Batista et al., 1999), from the African running frog, *Kassina sengalensis* (kassinateurin; Mattute et al., 2000). Most amphibian peptides show broad-spectrum antimicrobial activity against bacteria, fungi, and protozoa (Mignogna et al., 1998). Interestingly, D-amino acids have been found in some linear amphibian AMPs. For example, bombin H7 exhibits a D-leucine and bombins H3, H4, and H5 a D-allo-isoleucine at the same position. This modification reduces proteolysis and the antibiotic spectrum (Mignogna et al., 1993). Furthermore, amphibians share a lot of genes involved in mammalian immunity (Miele et al., 2000). For example, in many amphibians such as *Bombina* spp. and *Phyllomedusa bicolor* there is a correlation in nuclear-factor (NF-κB) and nuclear factor interleukin 6 consensus sequences related to the modulation of gene expression of their AMPs. These insights suggest that AMPs are well-preserved in all eukaryotes (Zhang et al., 2021). Defensins were identified and characterized in 1987 by Selsted's group from guinea pig neutrophils and subsequently isolated in several mammals (Selsted & Hakwig, 1987). Mammal defensins are small cationic peptides with highly conserved residues, typical of phagocytic cells, containing a specific pattern of cysteines with three disulfide bonds (Taylor et al., 2007; Yamashita et al., 1995). Mammalian defensins can be divided into three main classes as per their secondary structure: α-defensins, β-defensins, and cyclic θ-defensins. Human α-defensins are produced in the

small intestine by epithelial granulocytes and are chemoattractant for T cells (Chertov et al., 1996); Human-β-defensins (HBD), located on the skin and mucosal surfaces, chemotactically mobilize human dendritic cells, monocytes, and T cells (such as T cell CCR6), linking innate and adaptative immunity (Yang, 1999). Instead, θ-defensins are pseudo-cyclic defensins found in rhesus macaques but not in humans (Falanga et al., 2017). The secondary and tertiary structures in these three families bestow different antimicrobial activities. θ-defensins as cyclic peptides are extensively described below. HBDs are active against Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* and against the yeast *Candida albicans*. Among them, HBD-3 (Table 2) shows the strongest and broadest bactericidal activity: indeed, it is also active against Gram-positive bacteria, including multi-resistant *Staphylococcus aureus* strains and vancomycin-resistant *Enterococcus faecium* (Zelezetsky & Tossi, 2006), with an in vitro MIC of ~6 mg/ml in *Escherichia Coli* and in *Candida Albicans*, ~12 mg/ml in *Staphylococcus Candida aureus*. HBD 1–3 share similar tertiary structures consisting of three β-strands, held together by three intramolecular disulfide bonds, giving an antiparallel β-sheet near an α-helix at the N-terminal of the molecule.

NMR studies of HBD 1–3 demonstrated their similar tertiary structure despite their low sequence conservation. In HBD, disulfide bridges stabilize β-sheet structures, while a strictly conserved glycine residue warrants high conformational flexibility (Bauer et al., 2001). Due to its remarkably strong antibacterial activity, HBD-3, with a net charge of +11, inducible expressed by several human epithelial cells, triggered great interest. Researchers laid the basis to understand the SARs of β-defensins in order to find clues on how modifications of HBD-3 may enhance its bactericidal activity, antibacterial spectrum, and its salt tolerance. Sakagami-Yasui and colleagues reported that two arginine residues in the C-terminal region of HBD-3 are crucial for its antibacterial activity and salt tolerance (Sakagami-Yasui et al., 2017). Moreover, in 2018 Jiang et al. demonstrated the importance of the N-terminal portion of HBDs on its remarkable bactericidal activity (Jiang et al., 2018). Lastly, in recent studies Wj and colleagues combined HBD-3 and HBD-4 chimerically to further increase their antibacterial activity, proposing them as novel therapeutic antimicrobial agents (Wj et al., 2021).

Other linear peptides worth mentioning are cathelicidins (found in mammals), ceratotoxins (derived from insects), bombinin H (*H* for hemolytic and hydrophobic), dermaseptin, and magainins (derived from the skin of amphibians), all with an α-helix conformation (Bin Hafeez et al., 2021).

Bombinin H, isolated from amphibians' skin secretion (i.e., Bombinin H7, net charge +1) represents a family of peptides containing a D-alloisoleucine or a D-leucine in the second position of the sequence that influences their target specificity (Mangoni et al., 2000). Indeed, the D-isomer forms showed higher activity with respect L-isomer against the Gram-positive *Bacillus megaterium* and *S. aureus*, and against the Gram-negative *Yersinia pseudotuberculosis* and *P. aeruginosa* (Mangoni et al., 2000).

Cathelicidins, named in this way because of their high homology to cathelin-like domains, were discovered in mammalian bone marrow myeloid cells, and are also called "myeloid antimicrobial peptides" (MAP; Bals & Wilson, 2003). Moreover, bactenecins and prophenin, both derived from cathelicidins, are proline and arginine-rich mammalian peptides (Ho et al., 2016). PMAP-23 and indolicidin are tryptophan-rich mammalian peptides, also derived from cathelicidins (Yang et al., 2006). SMAP-29, one of the most studied cathelicidins and most powerful AMPs, comprises a N-terminal amphipathic α-helix and a C-terminal amidated region (Bagella et al., 1995; Zanetti et al., 1995). Usually, cathelicidins are tested in vitro at ~50 ng/ml, a higher concentration than physiological levels (Rowe-Magnus et al., 2019).

Peptide LL-37 (Table 2) is the only cathelicidin identified in humans (encoded by cathelicidin gene CAMP), released by its precursor human cationic antimicrobial protein-18 (Hcap18) and playing a major role in adaptative immunity: it is active against both Gram-positive and Gram-negative bacteria and features an antibacterial activity correlated to the extent of its α-helix conformation (Johansson, 1998).

Pro-LL-37 is cleaved by proteinase 3 enzyme to generate the active peptide LL-37. As AMP, LL-37 shows moderate antimicrobial activity against several pathogens (*Pseudomonas*, *Escherichia*, *Staphylococcus*, and *Enterococcus genera), but some proteases can be secreted by bacteria to promote LL-37 degradation. However, recombinant cathelin has been demonstrated to be more active* against bacterial strains resistant to LL-37 at concentrations of 16–32 μM in vitro (Zaiou et al., 2003). This means that modifications in the terminal -COOH of LL-37 could tune the proteolytic process and its activity.

However, as an antimicrobial agent, LL-37 requires ca. 10-fold higher concentrations to reach the MICs for killing Gram-positive bacteria, and studies at non-physiological levels to obtain appropriate antimicrobial activity are required (Rowe-Magnus et al., 2019). *LL-37* shows also immunomodulatory properties involved in acute and chronic inflammation, that is, LL-37 is augmented in the presence of IL-1b granulocyte-macrophage colony-stimulating factor and suppressed with IFN-γ, IL-4, or IL-12 (Fabisiak et al., 2016).

Lastly, a synthetic β-sheet forming short amphiphilic peptide IK8L (IRIKIRIK) designed by Ong and colleagues showed potent antimicrobial activity against reference strains of *Staphylococcus aureus* and MRSA: nonetheless, authors recommended that further studies

are required to investigate the activity of its derivatives (Ong et al., 2014).

While chemical synthesis has been used to produce most of the abovementioned molecules, the production cost for defensins is still too high, and synthetic defensins analogues are needed (Bindra et al., 2022). Some modifications were carried out in the N-terminal amphipathic domain and in C-terminal hydrophobic groups of cecropins. These changes included synthetic analogues of the naturally-occurring cecropins, such as hybrids of cecropin A (from Drosophila, net charge +7) and melittin (Wade et al., 1990) or a chimeric cecropin A–cecropin D (Andreu et al., 1992; Boman et al., 1989; Fink et al., 1989). Authors changed peptide length, reversed the order of residues, or turned the helix into a left-handed sequence containing D-enantiomers. Synthetic cecropins with *all*-D enantiomers formed left-handed helices resistant to proteases and with similar antibacterial activity as the naturally occurring L-enantiomers. It was demonstrated that their antibacterial activity is guaranteed by the presence of aromatic conserved residues in position 2, such as tryptophan or phenylalanine (Sato & Feix, 2006).

Cyclic peptides

Cyclic peptides are considered promising scaffolds for biopharmaceuticals (Gang et al., 2018) and as powerful agents against antibiotic resistance (Falanga et al., 2017). In the last two decades, several studies on cyclic antibacterial peptides were reported, likely because their favourable pharmacological properties make them very attractive therapeutic compounds. (Oh et al., 2014). Indeed, cyclic peptides have a crucial role in novel antibiotics development thanks to their higher stability under physiological conditions, higher membrane permeability, and greater oral bioavailability than linear analogues. Also, their reduced costs of synthesis, low toxicity, and, especially their better activity, make them promising candidates as novel antimicrobial agents.

Peptide cyclization confers high structural stability and, in turn, stronger binding to the target molecules (Joo, 2012). Furthermore, head-to-tail cyclic peptides are more resistant to endo and exopeptidases hydrolysis compared to linear peptides (Góngora-Benítez et al., 2014). Moreover, they have greater potential with enhanced activity in developing novel antibacterial agents (Dong et al., 2019).

Cyclic hexapeptides rich in tryptophan and arginine as cyclo-RRRWFW feature antibacterial activity against Gram-negative and Gram-positive bacteria and low hemolytic activity (Scheinpflug et al., 2013, 2015).

Bacitracin is a natural antibiotic cyclic peptide produced by *Bacillus subtilis*. Bacitracins are hydrophobic structures, comprising peculiar moieties such as D-amino acids, intramolecular thioether bonds and disulfide bonds (Caulier et al., 2019). However, the presence of disulfide bonds makes it difficult to develop synthetic cyclic analogues.

Bacitracin A is formed with a side chain-to-tail cyclization, where a bond is formed between a lysine side chain and the C-terminus (Figure 1). It is most active against Gram-positive bacteria and interacts with peptidoglycans in bacterial cell-wall biosynthesis (Siewert & Strominger, 1967). Currently, Bacitracin A is used in clinics as a topical antibiotic.

Other cyclic antibacterial peptides include bacteriocins that, instead, are backbone-cyclized peptides. Bacteriocins mainly show antibacterial activities against Gram-positive bacteria by means of cell membrane disruption (Cascales & Craik, 2010). Bacteriocin AS-48 features a cyclic backbone, a +6 net charge, and is secreted by the bacterium *Enterococcus faecalis*.

Subtilosin A is produced by *Bacillus subtilis* and is constituted by a head-to-tail cyclized backbone. Subtilosin A has three unusual (no disulfide bonds) covalent intramolecular cross-links formed post-translationally:

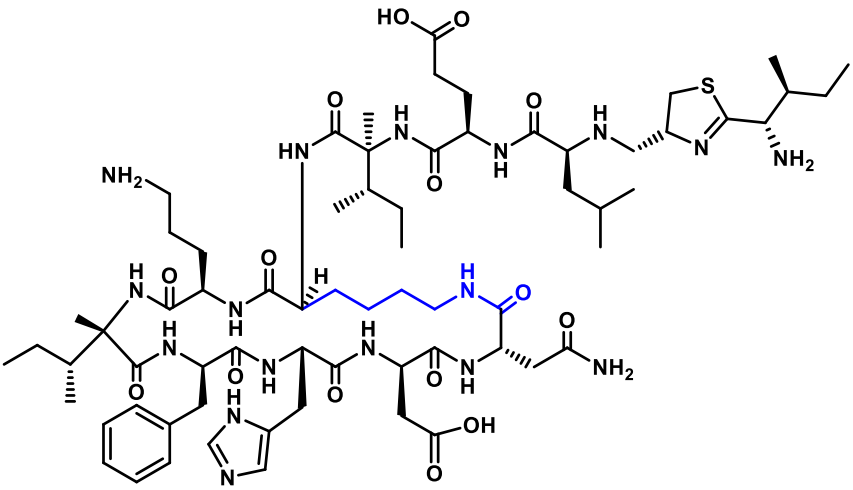


FIGURE 1 Chemical structure of bacitracin A: the side chain-to-tail closure is highlighted in blue.

it resembles a hairpin with hydrophobic residues exposed to the solvent (Arnison et al., 2013). Aureocin A-53 (Table 2) is an amphiphilic peptide rich in tryptophan and with +8 net charge (Netz et al., 2002).

Recent advances in novel bacteriocins isolated from *Pseudomonas* sp. strain 166 showed significant antimicrobial activity against *P. multocida* (Wang et al., 2022; Wang, Haqmal, et al., 2022). In particular, bacteriocin PA166 displayed a minimum bactericidal concentration between 2 and 8 μg/ml, placing good safety, therapeutic effect and physicochemical stability as a potential substitute for actual antibiotics.

Lanthipeptides, also named lantibiotics, are post-translationally modified cyclic peptides: their final form derives from the dehydration of serine and threonine residues, followed by the intra-molecular Michael-type addition of a 2,3-didehydroalanine or (Z)-2,3-didehydrobutyrine on a cysteine residue (Scheinpflug et al., 2013).

Subtilin and Nisin-A are typical lantibiotics belonging to the ribosomally synthesized peptide family, containing the rare amino acids dehydroalanine, dehydrobutyrine, meso-lanthionine, and 3-methyl-lanthionine (Entian & Vos, 1996): Nisin-A binds to the lipid II cell wall precursor and forms pores in the bacterial membranes (Khosa et al., 2016; Sakshi et al., 2016).

Recently, researchers reported the discovery of θ-defensins, cyclic octadeca-peptides featuring structures different from other defensins, expressed in the bone marrow and in leukocytes of rhesus macaques (Selsted, 2004). They are formed by two β-sheet forming peptides, connected by three disulfides bridges, and covalently bonded at their terminals, yielding cyclic molecules. They are active against Gram-positive and Gram-negative bacteria, but they also show strong activities against HIV and influenza A (Doss et al., 2009).

Ghadiri and colleagues pursued alternative strategies like alternating D- and L-amino acids in cyclic peptide sequences. These compounds adopt a planar ring conformation with the amide groups exposed to the solvent and available for hydrogen bond formation (Ghadiri et al., 1994; Granja & Ghadiri, 1994). The resulting self-assembled tubular structures (nanotubes) were reported to adopt a parallel orientation along the surface of biological membranes, disrupting the electrical membrane gradient and causing cell death (Fernandez-Lopez et al., 2001; Sanchez-Quesada et al., 2002).

Lastly, other cyclic peptides approved and used as antibacterial drugs are vancomycin (that will be discussed in further detail below), its synthetic lipoglycopeptidic derivative telavancin, and gramicidin (Table 1). Gramicidin S is a broad-spectrum cyclic antibacterial peptide with an in vitro MIC of 8 mg/ml in *Escherichia coli* and 2 mg/ml in both *Candida albicans* and *Staphylococcus aureus* (Hancock, 2001).

Lipopeptides

Lipopolipeptides, formed by peptides conjugated with fatty acid chains, are amphiphilic molecules, that usually self-aggregate into nanostructures (Dong et al., 1987; Hamley, 2015).

Vancomycin is a natural cyclic lipopeptide discovered in 1950 by Lilly in *Nocardia orientalis* and today is used for oral treatment against Gram-positive bacteria (Miao et al., 2005).

Recently, the cyclic lipopeptide daptomycin (Table 2 and Figure 2) was approved by FDA for the topical treatment of infections caused by Gram-positive bacteria.

It causes dissipation of the membrane potential of bacterial membranes due to its conformational changes (induced by binding to extracellular calcium ions) and increment of amphipaticity able to form transmembrane structures with subsequent disruption on membrane integrity (Chen et al., 2014; Jeu & Fung, 2004).

Daptomycin (Figure 2; Table 2) has a bactericidal effect against MRSA and MSSA, causing bacterial death in less than 1 h (Steenberger et al., 2005). Daptomycin was reported to be an antibacterial approximately 100 times more effective than Vancomycin on a hamster model of *Clostridium difficile* (Dong et al., 1987). Its antibacterial activity is strictly concentration-dependent (Wenzler et al., 2016), and requires complexation with calcium (Gregoire et al., 2021). Because it is not absorbed from the gastrointestinal tract, it has to be administered parentally. Its area under the curve/MIC (AUC/MIC; useful to evaluate the rate of bacterial killing) parameter of pharmacokinetics/pharmacodynamics (PK/PD; an alternative method of the classical dose-effect analysis for the drug effects evaluation) suggests that daptomycin distribution is limited (volume of distribution of 0.1 L/kg in healthy volunteers): this is due to its negative charge at physiological pH and its ~90% binding to plasma proteins (Schneider et al., 2017).

An important study on Daptomycin dose optimization versus renal functions allowed for the optimization of its renal clearance, and such values are newly recommended for dialyzed patients (Yılmaz & Özcengiz, 2016). Colistin (Table 1), also called Polymixin E, is a cyclic lipopeptide first isolated from *Bacillus polymyxa*, and clinically approved in 1950 for the treatment of acute and chronic infection of Gram-negative bacteria (MIC of 1–2 mg/L or less). It is the only antibiotic active against multidrug-resistant organisms (MDRO) *Acinetobacter* spp. and *P. aeruginosa* resistant to other antibiotics (Cunha & Opal, 2020). It acts with a bactericidal effect by interacting with the cytoplasmatic membrane displaying a cationic detergent activity and disrupting the lipid components of the membrane. However, its use is limited due to its nephrotoxicity, and it should be administrated with a

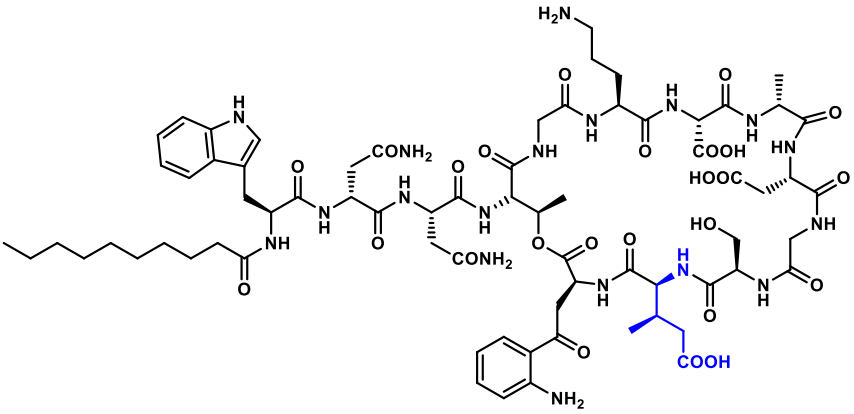


FIGURE 2 Chemical structure of Daptomycin. The (2S,3R)-MeGlu residue, highlighted in blue, plays a key role in its antimicrobial activity.

daily intravenous loading dose, meant as the average steady-state plasma concentration of colistin (Wang et al., 2022; Wang, Haqmal, et al., 2022).

MECHANISMS OF ACTION

Over the last decades, considerable efforts were undertaken to better grasp the mechanisms of action of AMPs. These mechanisms have been extensively reviewed recently, but they are not yet completely understood (Nguyen et al., 2011; Sani & Separovic, 2016; Udaondo & Matilla, 2020; Wang et al., 2009) Regardless of their source, AMPs actively target lipid membranes in order to disrupt their integrity (Sani & Separovic, 2016). As such, their mechanisms of action are strongly dependent on the composition of lipid membranes. Defensins, cecropins, bactenecins, dermaseptins, and magainins have shown a direct correlation between their activity and trans membranal permeabilization capability, likely related to their electric charge distribution, and suggesting the importance of the cationic character of effective AMPs to alter the membrane barrier (Madani et al., 2011). Examples of anionic Asp-or Glu-rich antibacterial peptides suggest more complex mechanisms of action that still have to be clarified (Harris et al., 2009). Anionic peptides with antibacterial activity were found in the pulmonary secretions of ruminants such as sheep and cattle (Caverly et al., 2001). These peptides have shown activity against both Gram-positive and Gram-negative bacteria (minimum bactericidal concentration, MBC > 600 μM), but, in particular, against the Gram-negative bacteria *Mannheimia haemolytica*: activity increased in the presence of Zn^{2+} (MBC < 60 μM; Brogden et al., 1996). These data point out the possible therapeutic use of surfactant-associated anionic peptides as an antimicrobial agent to treat *M. haemolytica* (Caverly et al., 2001).

Others described different antimicrobial mechanisms of peptides, for example, selective binding to intracellular targets (and not to bacterial membranes) to cause a perturbation of cell homeostasis or direct inhibition of protein synthesis, yielding to apoptosis (lactoferricin and hybrid cecropin-melittin; Wang, 2014; Yoo et al., 1997), or autolysis (lantibiotic ninisin-A; Sahl, 1994).

Cathelicidins preferentially kill Gram-negative bacteria in vitro but engineered cathelicidins rapidly target Gram-negative and Gram-positive bacteria by penetrating their cytoplasmic membrane. A recent study (Rowe-Magnus et al., 2019) described the production of reactive oxygen species as the primary effect of cathelicidins, revealing general damage against which bacteria might evolve resistance mechanisms.

Lastly, different models were introduced to explain the main mechanism of action of antibacterial peptides, such as membrane permeabilization (Galdiero et al., 2015; Shai, 1995), carpet model (Pouny et al., 1992), barrel-stave model (Shai, 1999) and toroidal-pore model (Matsuzaki et al., 1996). Such mechanisms are described considering the peptide position referred to the membrane target, but further insights still are required.

Understanding the chemical structures and subsequent modifications, as well as functional groups to prevent proteolysis or bacterial deactivation, are essential to investigate how antibacterial peptides act in their mechanisms.

STRUCTURE–ACTIVITY RELATIONSHIPS

AMPs are considered one of the best solutions as alternative antibiotics, thanks to their broad-spectrum and low cytotoxicity (Liu et al., 2019).

Due to the remarkable diversity of the structural conformations of antibacterial peptides, drawing common SARs is a huge task still far from being completed, and several aspects should be considered to design and investigate novel agents. Still, some guidelines based on the peptide sequences, have been found to be crucial for peptide antibacterial activity. For example, hydrophobicity patches and positively charged residue action are very often typical features of effective antibacterial peptides (Yan et al., 2021).

The role of internal disulfide bridges

Intra-molecular disulfide bridges are common post-translational modifications found in natural antibacterial peptides and researchers suggest they are important structural features for their biological activity. For instance, a disulfide bridge is necessary for the antimicrobial activity of these peptides (Lei et al., 2019).

In defensins, their presence was demonstrated to be necessary for their antibacterial activity (Krishnakumari et al., 1999; Reddy et al., 2004). The presence of two/three disulfide bridges is essential to preserve the β-structuration in β-defensins (White et al., 1995). Moreover, disulfide bridges play an important role in the activity of short peptides like tachyplesins (Matsuzaki et al., 1997) and protegrins (Harwig et al., 1998). Tachyplesin I, is a cyclic peptide making antiparallel β-strands stabilized by two disulfide bonds: it shows strong membrane permeabilization of bacteria due to the formation of anion-selective pores (Matsuzaki et al., 1997). Lanreotide and Pasireotide are disulfide cyclic octapeptides, two synthetic derivatives from somatostatin, used for the treatment of acromegalia and endocrine tumours (Zorzi et al., 2017). Romidepsin is a depsipeptide with a bicyclic structure obtained by a disulfide bond. It is a natural product isolated from Gram-negative *Chromabacterium violaceum* approved for the treatment of T-cell lymphomas (Jain & Zain, 2011). Furthermore, its unique structure allowed it to be clinically approved, as histone deacetylase (HDAC) inhibitor. Indeed, its disulfide bond, which acts as a pro-drug (more stable than its corresponding drug) in presence of reducing agents, is opened to provide a free thiol that chelates zinc ion in the active site of HDAC enzyme modulating gene expression (Rath et al., 2010). In general, therefore, a disulfide bridge changes the conformation ensuring ultra-stable structures and may increase the affinity to recognize the specific target (D'Souza et al., 2014).

To be mentioned is, moreover, the crucial role of disulfide bond in AMPs conjugation with aminoglycosides. For example, Chmielewski group conjugated with a disulfide bond the antimicrobial CAPH P14LRR with Kanamycin (P14KanS) demonstrating excellent antimicrobial activity with enhanced MIC values against ESKAPE pathogens compared to Kanamycin alone (Mohamed et al., 2017).

Repeats of lysine, arginine, and histidine

The abundance and the repetition of some amino acids in AMPs influence their biological activity. Natural antibiotics, especially AMPs, show repeated sequences of poly-amino acids bestowing excellent antibacterial properties (e.g., cationic properties, enhanced cell membrane interactions, etc.).

Lysine and arginine are responsible for AMPs cationicity at physiological pH (Epand et al., 2003): they provide strong electrostatic interactions with anionic lipids of Gram-negative bacteria membranes and with cell walls of Gram-positive bacteria (Zheng et al., 2021).

In 1977 the first natural lysine-rich antibacterial polymer was found in *Streptomyces albulus* (Shima & Sakai, 1977). α-polylysines are formed by condensation between the α-amino group and α-carboxylic groups of repeated lysine residues (Katchalski et al., 1947).

Clusters of charged residues confer a bactericidal capacity due to their binding to the hydrophobic core of the lipid bilayer, thus inducing a partition (Gopal et al., 2013). However, also peptide chain length and amphipathicity influence their bacterial membrane permeabilization propensity. In other words, Lysine-rich peptides may depolarize bacterial cell membranes through electrostatic interactions, causing membrane disruption and bacterial death (Xi et al., 2016). However, it has been widely demonstrated that, despite their excellent antibacterial activity, high amounts of lysine increase cytotoxicity as well (Xi et al., 2016).

Du and colleagues introduced α-polylysine based micelles to maintain a high surface potential: micelles join the external bacterial membrane by electrostatic interactions, and cause a mild disruption of its outer membrane, resulting in an equally active but less cytotoxic compound (Xi et al., 2016).

ε-Polylysine AMPs are characterized by a peptidic bond formed between the gamma-amino functional groups of lysine residues and carboxyl groups (Hiraki, 2001). Studies demonstrated that ε-polylysine is more active than α-polylysine and their antibacterial activity is enhanced with peptides featuring 10 or more lysine residues (Shima et al., 1984).

Polylysine polymers are excellent biocompatible carriers, suited for tissue engineering applications, in the wound healing process, and as functionalized electrospun materials. Electrospun polylysine nanofibers have also shown increased antibacterial properties and selective toxicity against tumour cells (Patil & Kandasubramanian, 2021).

Polyarginine polymers were first tested against *Escherichia coli* and *Staphylococcus aureus*. Their mode of action is the depolarization of the plasma membrane cell causing lysis (Li et al., 2013; Sepahi et al., 2017). Indeed, pH modulations of plasma membranes enhanced antibacterial activity and toxicity against bacteria.

On the other hand, the bactericidal activity of polyhistidine polymers is effective in acidic conditions, allowing for their interactions with the anions of cell membranes (Holdbrook et al., 2018). The mechanism of action of polyhistidine peptides relies on the translocation of CPP across cell membranes (Guo et al., 2016; Lee et al., 2019). Although the exact mechanism of CPP internalization remains to be elucidated, they seem to

interact with cell membrane proteins causing the subsequent destabilization of their binding sites and the entrance of the peptide into the bacteria. Functionalization with polyhistidine can be convenient in drug delivery approaches for biomedical and biotechnology applications, as well as a glycosyl moiety can be used in CPP-conjugates delivery.

Glycosylation

It is the authors' opinion that the importance of glycosylation on antibacterial peptides is considerably underestimated. Glycosylation is one of the most relevant post-translational modifications, bestowing diversity and specificity to protein functions (Bednarska et al., 2017). *O*-glycosylated proline-rich antibacterial peptides were found and studied in insects (diptericin, drosocin, formaecin, and pyrrhocoricin) and in bacteria, such as *Lactobacillus plantarum* (glycocin F and enterocin F4-9; Talat et al., 2011; Wang, 2012).

It was demonstrated that antibacterial activity decreases together with glycosylation reduction (Strub et al., 1996), suggesting a different mechanism of action from the typical membrane permeabilization effect of other antibacterial peptides. Unfortunately, such a mechanism of action is still largely unknown, but recent works suggest that peptidoglycan biosynthesis and/or peptide-receptor recognition pathways may play a major role in their activity (Mackintosh et al., 1998).

Vancomycin and teicoplanin are amphoteric glycopeptides naturally synthesized respectively in *Streptococcus orientalis* and *Actinoplanes teichomyetius*, the latter showing a fatty acid tail. They share an aglycon heptapeptide backbone and a peptide portion constituted by non-proteogenic amino acids, while their carbohydrate portion consists of simple sugars and amino-sugars. The molecular structures of vancomycin and teicoplanin are conformationally rigid due to their macro-cycled systems and ethereal bridges (Recktenwald et al., 2002). In Figure 3 the chemical structure of vancomycin is shown, with an unusual D-Ala residue interacting with peptidoglycan synthesis and interfering with the formation of the bacterial cell wall.

Nonetheless, some bacteria have developed resistance to the extensively used vancomycin and teicoplanin antibiotics: the emergence of vancomycin-resistant enterococci is a serious concern for clinical treatments of Gram-positive bacteria (Butler et al., 2013). Clinical studies and meta-analyses showed that continuous infusion of vancomycin achieved target concentration efficiently with lower nephrotoxicity (Ampe et al., 2013). However, the maximum dosage clinically applicable of vancomycin against two MRSA strains was not effective and, probably, an AUC/MIC of 400 μg.h/ml is inefficient to fight *S. aureus resistance* (Lenhard et al., 2016). Indeed, it is important to note that increased dosages required for resistant strains could lead to augmented nephrotoxicity.

Teicoplanin (Figure 4) is used against Gram-positive bacteria, and it is less toxic than vancomycin. A recent study on the pharmacokinetic of teicoplanin demonstrated that a target AUC/MIC of ≥900 μg.h/ml is bacteriologically efficient against MRSA infections (Matsumoto et al., 2016). Other natural and synthetic antibacterial glycopeptides are balhimycin, dalbavancin, telavancin (Table 1), but also ristocetin and ramoplanin (Tiyanont et al., 2006; both ones of limited use due to their toxicity). Dalbavancin (Figure 4; Table 1) is a semi-synthetic derivative of teicoplanin. It was approved by FDA for acute bacterial skin and skin structure infections. It is active against Gram-positive bacteria including MRSA, with a MIC of <0.125 μg/ml. It has a higher potency and extended half-life of over 1 week (due to its long

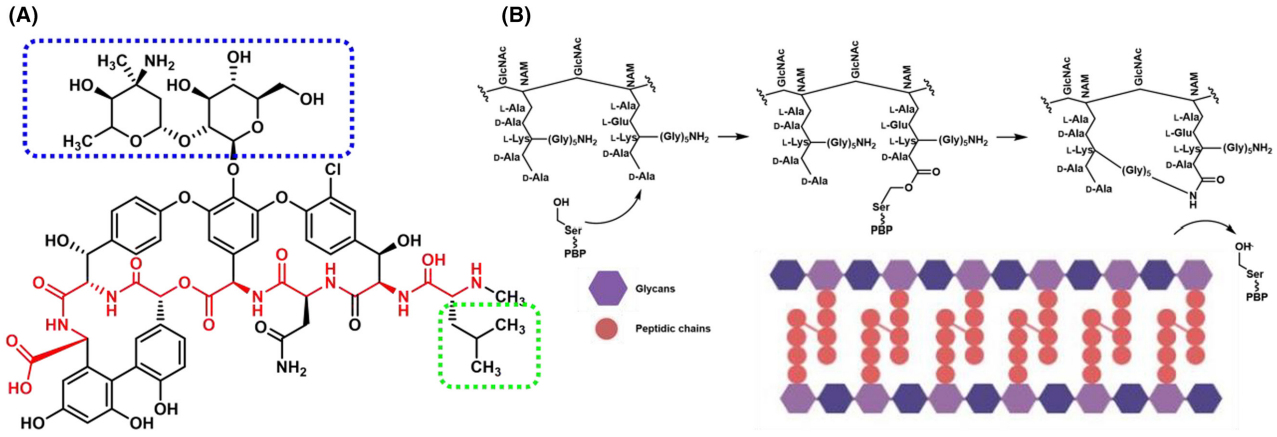


FIGURE 3 (A) Chemical structure of vancomycin. In red is highlighted the heptapeptide backbone: the dotted blue square highlights its glucidic portion; the dotted green square contains the unusual D-Ala, involved in bacterial resistance. (B) The extracellular stage of peptidoglycan biosynthesis. In red is highlighted the D-Ala-D-Ala motif. Vancomycin's antibacterial activity is correlated to its interaction with carboxy-terminal D-Ala-D-Ala muramyl pentapeptides during peptidoglycan biosynthesis (Allen et al., 1996; Hammes & Neuhaus, 1974). GlcNAc, acetylglucosamine; NAM, *N*-acetilmuramic acid.

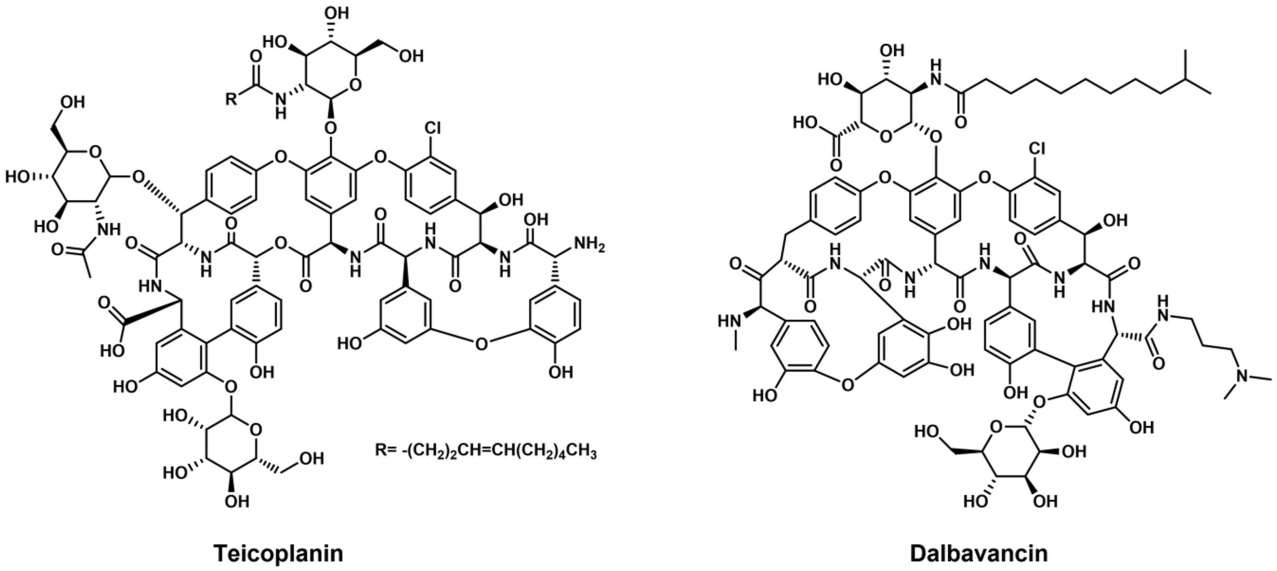


FIGURE 4 Structures of teicoplanin and dalbavancin.

lipophilic side-chain) that allows weekly administrations (Leighton et al., 2004).

Oritavancin (Figure 5; Table 1) is a semi-synthetic derivative of vancomycin synthesized by Eli Lilly and Co. and approved in 2014 for clinical use with a single dose of 1200 mg infused over 3 h in adult patients for the treatment of ABSSI caused by Gram-positive bacteria including MRSA (Rosenthal et al., 2018). In in vitro tests, oritavancin showed antibiofilm activity against *S. aureus* strains with a MIC of 0,5–4 μg/ml. Moreover, Oritavancin (but not Dalbavancin) is active against *VanA-type Enterococci* resistant to other treatments (MIC of 2–8 μg/ml), and it is considered more tolerable and safer than other lipoglycopeptides (Roberts et al., 2015; Stein & Saravolatz, 2015). Although *Enterococcus* and *Staphylococcus* species developed resistance to vancomycin, recently several strategies to overcome vancomycin resistance were studied, and promising derivatives have shown improved efficacy (Zeiders & Chmielewski, 2021). For example, vancomycin was conjugated with a CPP composed of eight arginine residues resulting in a CPP-conjugated glycopeptide (Vancomyncin-D-octaarginine conjugate, V-r8). V-r8 showed comparable activity to Vancomycin alone but cleared ~95% of MRSA biofilms at 80 times its MIC in vitro (Antonoplis et al., 2018). Others (Ruczyński et al., 2019) conjugated Vancomycin in two different positions, the C-terminus and the vancosamine portion, with PEG tethers, showing in all conjugates enhanced antibacterial activity against resistant *S. aureus* and *E. faecium*.

N- and C-terminal capping

N- and C-terminal modifications are post-translational features that usually refer to N-terminal acetylation and C-terminal amidation respectively.

C-terminus amidation inhibits hydrolysis from carboxypeptidases and usually stabilizes H-bonds of α-helices acting against bacteria. Indeed, carboxypeptidases are enzymes that selectively hydrolyze C-termini and release the peptide chains.

In maximin H5, an anionic AMP from amphibians, C-terminal amidation resulted to be crucial for its antibacterial activity: its deamidation triggered a structural perturbation, causing a decreased presence of α-helices required for interaction with membrane bacteria (Dennison et al., 2015).

In some short peptides, acetylation of the N-terminus decreases compound efficacy against *P. aeruginosa* and *S. aureus* but does not affect antibacterial activity against *C. albicans* and gentamicin-resistant MRSA (Saikia et al., 2017).

Other terminal modifications encompassing N-terminal lipidation (introducing fatty acid chains; Datta et al., 2016), C-terminal "Rana box" (a C-terminal cyclic heptapeptide), and helix-capping motifs at the helix termini (specific patterns of hydrogen bonding and hydrophobic interactions; Park, 2004) can enhance both the antibacterial potency and the range of action against bacteria.

Replacements with D-amino acids

In general, the substitution with D-amino acids is a method to increase the antimicrobial activity (Gan et al., 2021) of cationic amino acids with similar amphipathicity (Hamamoto et al., 2002). Replacements of peptide sequences with D-amino acids is an effective strategy to prevent their proteolytic degradation: for example, the D-enantiomeric analogue of pleurocidin is resistant to trypsin, plasmin, and carboxypeptidase

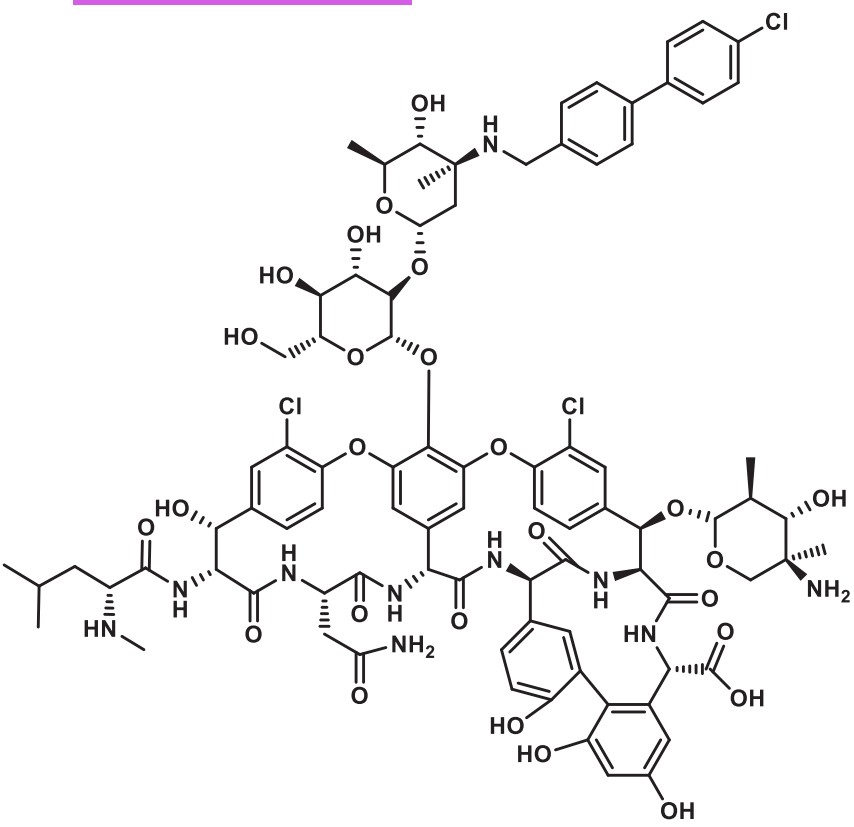


FIGURE 5 Chemical structure of Oritavancin.

(Jung et al., 2007; Kapil & Sharma, 2020). Lu et al. (2020) reported that both D- and unnatural amino acids improve the stability of the peptides against protease-mediated hydrolysis. Others (Hong et al., 1999) studied the effect of D-amino acids substitution in several diastereomers of KKVVFKVKFKK sequence (called KSLK), a depsipeptide with broad antibacterial and antifungal activity. D-amino acid substitution at one or both termini formed left-handed α-helixes and increased the overall antimicrobial activity: conversely, D-amino acid substitution in the middle chain resulted in activity loss.

In general, since D-amino acids resist proteolytic cleavage, punctual substitutions of the proteolytic cleavage site warrant the easiest method to improve the stability of AMPs without affecting their activity (Kapil & Sharma, 2007).

It was also demonstrated in vivo experiments that D-substitutions of bombinins H improved their antibacterial activity: indeed, additions of D-amino acids showed more potent antimicrobial activity and cell selectivity than its respective L-isomer-only analogue (Mangoni, 2013).

Self-assembling peptides

Self-assembling is a ubiquitous nanostructural phenomenon that occurs in conditions of thermodynamic equilibrium. Self-assembling peptides self-organize (thanks to hydrogen bond formation, hydrophobic interactions, π–π stacking, and electrostatic interactions) in well-ordered structures that include fibrils, micelles, vesicles, nanotubes (Mandal et al., 2014).

Being self-assembly directly correlated to the formation of specific secondary structures, these compounds can be designed to be promising antibacterial agents (Chang et al., 2017). Indeed, self-assembling peptides can be tuned (in terms of amino acid sequences, chemical groups, spacer-moiety, charge, and amphiphilicity) to increase the antibacterial activity and to influence the interaction with the bacteria membranes.

For example, PTP-7 (FLGALFKALSKLL), a well-known AMP, do not self-assemble at physiological conditions, but substituting two Lysine with two Arginine residues confers significant self-assembling propensity (Chen & Liang, 2013).

Peptide self-assembling was demonstrated both in natural and synthetic AMPs (Oren & Shai, 2000): intriguingly, they feature both broad-spectrum antimicrobial and anti-inflammatory effects, but low toxicity against human cells (Tian et al., 2015).

The mechanism of action of self-assembly antibacterial peptides seems to be due to their incremental self-aggregation at the cell membrane as incubation time increases until lipidic membrane disruption takes place (Chen, Hu, et al., 2012; Chen, Patrone, & Liang, 2012). Pioneering research shows that positively charged residues interact with negative charges on bacteria cell membranes, followed by the insertion of the peptide into the hydrophobic cores of the membrane due to

self-aggregation, amphipathicity, and formation of hydrophobic patches (Chen et al., 2010).

KLD-12 (KLD) is a self-assembling peptide with a high potential for tissue engineering applications (Sun & Zheng, 2009). While KLD did not show any antimicrobial properties till 100 mM, N-terminus functionalization with arginine residues enhanced antibacterial activity and caused damage to the bacterial membrane of *Escherichia coli* (Tripathi et al., 2015).

Furthermore, the self-assembling phenomenon allows for high target selectivity: A(9)K(1) self-assembling peptide has been reported to be toxic for both bacteria and cancer cells, but not on non-tumoral cells (Chen, Hu, et al., 2012; Chen, Patrone, & Liang, 2012). Moreover, in A3K, A6K, and A9K amphiphilic peptides, antibacterial activity was showed to strongly correlate with peptide hydrophobicity, and the membrane permeation efficiency increased well together with peptide concentration and incubation time (Chen et al., 2010).

Another asset for self-assembling antibacterial peptides is their resistance to several proteases, probably due to the protection of cleavable sites taking place after their self-aggregation into supramolecular structures (Shima et al., 1984).

APPLICATIONS

Over the last 20 years, AMPs triggered increasing attention from the scientific community because of the increasing antibiotic resistance of microorganisms related to the excessive use of antibiotics worldwide (Willyard, 2017), from medicine to food preservation (Zhen et al., 2022), to the agriculture and animal husbandry. On the contrary, antibacterial peptide materials are less susceptible to drug resistance due to their different mechanisms of action and their chemical–physical features. Unfortunately, the production of natural antibacterial peptides still falls in the realm of high-cost compounds with significant production times concerning the expectancies of high clinical demand (Yan et al., 2022). Thus, optimization of their pharmacokinetic and pharmacodynamic characteristics is necessary for the design and synthesis of new feasible antibiotic candidates.

For instance, synergistic actions in combination with existing antibiotics can improve the efficacy of traditional antibacterial treatments: for example, the use of metals (antibacterial materials combined with titanium dioxide and silver nanoparticles; Xie et al., 2018), polysaccharides (triggering glycan polymerization; Kahne et al., 2005) and biomimetic peptides (mimicking host-defence peptides against bacterial resistance; Wei et al., 2020) are just a few ones of the multiple options available. Moreover, nanoparticles, used as smart delivery systems, can improve the pharmacokinetics, biodistribution, biosafety, and antibacterial effectiveness of AMPs (Zhang et al., 2021).

Carrier protein strategy

Solid-phase chemical synthesis is well positioned for the synthesis of large amounts of short and medium-length peptides. Many AMPs were also produced by recombinant cost-effective DNA technology (Li, 2009). However, on one side the expression of consistent amounts of peptides could show toxicity for the host itself, thus hampering its production, while the sensitivity of expressed peptides to proteases may be also a crucial issue. Recent approaches have fused the desired peptide to a carrier protein to avoid hydrolyzation and reduce toxic effects (Pane et al., 2016). The fusion mimics the native precursor of the peptide in its native form. The target peptide is expressed in the respective carrier-peptide junction. In this way, the peptide is protected from proteases and the host from toxicity (Vassilevski et al., 2008). Subsequently, the peptide is released by the carrier protein into the bacterial cell target. A carrier protein strategy was recently developed for dalbavancin by using a chimeric carrier-protein epitope (Economou et al., 2012).

Thioredoxin, a low-molecular-mass protein, was the most reported carrier protein for the fusion expression of AMPs (Li, 2009). Thioredoxin fusion technology is frequent as carrier fusion to promote the solubility of recombinant peptides and proteins in *E. coli* cytoplasm (LaVallie et al., 2000), and it was revealed an efficient strategy for AMPs in *E. coli*.

Peptide nucleic acids

Peptide nucleic acids (PNA) are oligonucleotides comprising a peptidic backbone and nucleic acids bases. They were first synthesized through solid-phase peptide synthesis as DNA/RNA analogues in which sugar-phosphate is replaced by *N*-2-aminoethylglycine units (Nielsen et al., 1991). Antisense PNA conjugates are promising compounds for new antimicrobials in drug discovery because of their resistance to nucleases and proteases and their peculiar RNA hybridization properties (Goltermann et al., 2019). PNA-based compounds can be realistically considered effective antibacterials because of their biocompatibility and their ability to penetrate tissues and cells while blocking bacterial expression (Goltermann et al., 2019).

A bactericidal effect was interestingly found in *Escherichia coli* for (KFF)3K peptide conjugated to a PNA targeting the essential bacterial gene *acpP* (Good et al., 2001). An optimal PNA sequence length for the maximization of antimicrobial efficacy was reported to range from 10 to 12 nucleobases. Various studies showed the abolishment of bacterial growth via an anti-filamentous temperature-sensitive mutant Z (ftsZ)-PNA (Han et al., 2021; Zhang et al., 2018) and the inhibition of biofilm formation with anti-efaA PNA (Narenji et al., 2020).

PNA can form duplexes, triplexes, and quadruplexes with DNA, but it can also bind RNA: it can inhibit gene expression, and, as a consequence, bacterial growth as well (Narenji et al., 2017). Antisense peptide-PNA conjugates have shown antimicrobial activity (Otsuka et al., 2017) due to PNA sequences suppressing essential genes for fatty acid biosynthesis (Ji et al., 2004) and cell wall biogenesis (Bosch et al., 2011). Inside bacteria, antisense PNA can influence ribosome activity by binding mRNA. Therefore, PNA-based antibacterials can be synthesized for selected bacteria (Wojciechowska et al., 2020), and some advances were made regarding PNA-AMPs conjugates involving the transport in a membrane protein SmbA (Ghosal et al., 2013).

Peptidomimetic approach

Peptidomimetics are designed to mimic natural peptides, but they may have peptide bond isosteres and have the ability to bind with their natural targets (i.e., the same biological effect). This technology may be useful as a part of medicinal chemistry optimization (Henninot et al., 2017). Indeed, peptidomimetics are a versatile strategy to overcome some limitations such as rapid proteolysis of natural peptides. Furthermore, antimicrobial peptidomimetics display antibacterial activity against drug-resistant strains, due to their reduced susceptibility to the development of bacteria resistance (Méndez-Samperio, 2014).

By fine-tuning their structure, it is possible discovering new drug candidates with higher proteolytic stability, higher bioavailability, and enhanced selectivity (Grauer & König, 2009). Several antimicrobial peptidomimetics developed include peptoids (Kapoor et al., 2011), β-peptides (Karlsson et al., 2010), β-turns analogs (Srinivas et al., 2010), arylamide oligomers (Hua et al., 2010), and the called AApeptides.

Monomethyl Auristatin E (MMAE) is an antimitotic agent that can be considered a peptidomimetic agent (Thundimadathil, 2012). MMAE is a synthetic derivative of natural analogue dolastatin, extracted from the sea hare *Dolabella auricularia*. As dolastatin is extremely cytotoxic, it cannot be used as a drug and, it was recently evaluated for antibody-drug conjugates and other tumour-targeted chemotherapy (Piarulli et al., 2019).

CONCLUSIONS AND PERSPECTIVES

Antibacterial peptides belong to the innate host defence system (Ganz, 2004; Tew et al., 2009) and a deep understanding of their structural characteristics is necessary to comprehend their mechanisms of action and functional activity. An updated selection of relevant websites in AMPs topics was reported by Wackett to highlight all quality and impacting developments in this field (Wackett, 2019). AMPs, as defence agents, are very common in nature and they follow similar lines of action, such as membrane penetration, regardless of their origin. This behaviour suggests that nature has encoded schemes of defence in fighting bacteria and that such organizations still need to be explored to better understand how to develop new antibiotic agents.

Elucidating the antibacterial properties of peptidic structures allows us to better understand their huge potential against diverse infections and, in general, multidrug bacterial resistance. To this purpose, medicinal chemistry, in a multidisciplinary synergic approach with organic synthesis and chemical biology, should provide alternative roads to novel affordable antibiotic drug candidates.

In this review, various classes of antibacterial peptides are presented paying special attention to their molecular structures. In particular, the provided insights on the SARs of these classes make a strong case for overhauling these molecules as leads in antibiotic drug discovery research and the design of new chemical tools against resistant infections.

Although new approaches, such as in silico predictions (Stokes et al., 2020), computer-based molecular design and high-throughput screenings, are widely used in drug discovery, nature-inspired products, and the understanding of their biological pathways are still promising routes for the development of effective compound libraries in the fight against antibiotic resistance. Lastly, due to their potential selective activity and mode of action that rivals existing antibiotics, the development of novel selective antibacterial peptides that can act synergically against multiple specific targets could become the new alternative to antibiotics in the future. Finally, as AMPs can be seen in different aspects, this review provides selected approaches together with crucial examples and it is not intended to be comprehensive of all discovered compounds. We focused on the discussion of naturally occurring, semi-synthetic, and synthetic chemical structures evaluable as starting points to re-emerge interest in this field.

AUTHOR CONTRIBUTIONS

Maria Gessica Ciulla: Conceptualization (equal); writing – original draft (equal); writing – review and editing (equal). **Fabrizio Gelain:** Conceptualization (equal); supervision (lead); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors declare no competing interests.

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