Antibacterial Peptides and Their Mechanism of Action

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Contributor: Tania Vanzolini

Despite the great strides in healthcare during the last century, some challenges still remained unanswered. The development of multi-drug resistant bacteria, the alarming growth of fungal infections, the emerging/re-emerging of viral diseases are yet a worldwide threat. Since the discovery of natural antimicrobial peptides able to broadly hit several pathogens, peptide-based therapeutics have been under the lenses of the researchers. Antimicrobial peptides generally affect highly preserved structures, e.g., the phospholipid membrane via pore formation or other constitutive targets like peptidoglycans in Gram-negative and Gram-positive bacteria, and glucan in the fungal cell wall. Additionally, some peptides are particularly active on biofilm destabilizing the microbial communities. They can also act intracellularly, e.g., on protein biosynthesis or DNA replication. Their intracellular properties are extended upon viral infection since peptides can influence several steps along the virus life cycle starting from viral receptor-cell interaction to the budding. Besides their mode of action, improvements in manufacturing to increase their half-life and performances are also taken into consideration together with advantages and impairments in the clinical usage. Thus far, the progress of new synthetic peptide-based approaches is making them a promising tool to counteract emerging infections.

Keywords: antimicrobial peptides ; antifungal ; antibacterial ; antiviral ; peptide-based therapies ; synthetic peptides

1. Introduction

All antimicrobial peptides (AMPs) share common features, such as a sequence composed of less than 100 amino acids (aa), [1] with the majority having between 10 and 60 aa [2]. Even if some anionic AMPs, rich in glutamic and aspartic acids, are negatively charged [3], almost all antimicrobial peptides have a net positive charge for the presence of a high number of lysine, arginine and histidine (protonated in acidic conditions) [4]. Finally, another common feature is represented by the hydrophobicity conferred by hydrophobic aa that often overcomes 50% of the total amino acid sequence [5]. The high lipophilicity is useful especially for the penetration in the biological membranes but considering the net charge, overall, AMPs are amphipathic molecules. The classifications are based on their structure or the presence/absence of recognizable motifs. AMPs could be α-helix, β-sheet, linearly extended, both α-helix and β-sheet, cyclic and with complex structure or, seen from a different perspective, tryptophan- and arginine-rich, histidine-rich, proline-rich and glycine-rich [6] [7].

In the last decades, the increasing resistance to antibiotic treatments, i.e., Methicillin, Vancomycin-resistant *Staphilococus aureus* and the rise of species with intrinsic multi-drug resistance, such as *Candida auris*, highlights the need for the development of new agents [8][9][10]. Studies on the AMPs synthetic analogs provided a new tool to understand the different and unique modes of actions against diverse microorganisms.

2. Synthetic Antimicrobial Peptides

Natural antimicrobial peptides have been always present during the evolutionary process [11], however, many natural AMPs showed host toxicity, rapid degradation by proteases, instability due to pH changes, loss of activity in presence of serum and high salt concentrations, lack of suitable delivery systems able to limit the drawbacks, and high costs of production [12][13][14]. Moreover, their complex design, low antimicrobial activity and pharmacokinetics led many laboratories to improve their structure and amino acid sequence to enhance their therapeutic properties [15]. Despite the multiple obstacles in the clinical application, synthetic peptides were developed to overcome the difficulties linked to the natural peptides while mimicking their pharmacological qualities [16].

The approaches commonly used for the development of non-natural AMPs are (1) the site-directed mutations characterized by the addition, the deletion or the substitution of aa, (2) the de novo design which doesn't use any template sequence, (3) the template-based design that uses fragments of the parental compound as starting point for the construction of new AMPs (in this case, antibodies seem to be a big source of patterns, especially those which recognize and bind components of the cell membrane and wall), and lastly (4) the self-assembly-based design that exploits the formation of simple nanostructures like dimers, or more complex as micelles, vesicles and nanotubes [2].

Semi-synthetic AMPs maintained the active sites of the natural source, but chemical changes were brought in order to reach the optimal properties whereas synthetic AMPs are obtained from chemical synthesis with frequent usage of the solid phase. This technique is based on the addition of one aa at a time, thus favoring the investigation of the role of each amino acid in the sequence [17].

Apart from the solid-phase method, synthetic AMPs can also derive from the catalytic ring-opening polymerization (ROP) of α-amino acid *N*-carboxyanhydride (NCA), an exquisite tool for the fabrication of long polypeptides with low polydispersity but variable chemical composition and topology [18]. Chemical synthesis represents a great step forward in peptide production with higher efficiency, reliability, and speed, especially when compared to the AMPs produced through the technology of the recombinant DNA followed by bacterial expression and purification.

The advances in the AMPs synthesis are the result of several studies about machine learning and algorithms able to predict or identify potential sequences based on the physicochemical and structural properties and on the quantitative structure-activity relationship (QSAR) of AMPs and targets already present in databases followed by high-throughput screenings [19]. Therefore, several strategies were tested to achieve a superior half-life e.g., the usage of D-amino acids [20], peptide cyclization [21], unnatural amino acids [22]. With peptidases able to recognize mainly L-amino acids sequences, stereogenic D-variants of amphipathic peptides could be resistant to proteolysis [23], as well as peptides with uncommon amino acids, i.e., ω and β-amino-acids [24][25]. Protection from cleavage could be also conferred by modifying or protecting vulnerable peptide bonds so that they cannot be easily accessed [26]. In some cases, such modifications could be applied just to the *N*- and *C*-terminus i.e., *C*-amidation or *N*-acetylation [27].

Similarly, PEGylation, the covalent attachment of polyethylene glycol (PEG) chains to lysine or to the N-terminus [28], could also be applied to mask other residues like arginine [29]. On the other hand, lipidation, consisting in the attachment of one or more fatty acid chains to a lysine residue or to the amine of the N-terminus, [30] could improve AMPs properties by enhancing their interaction with the membranes. Introduction of sulfonamide groups has been also investigated to exploit their bio-active properties, enhance their proteolytic stability and hydrogen bonding ability [31].

Another approach to improve the half-life of peptides in vivo is to synthesize them as dendrimers around a residue or a linear polymer core [32]. These multiple antigen peptides (MAP) developed by Tam and colleagues [33] are mainly constituted by a lysine core to which peptide chains are attached [34]. The number of bi-, tri-, tetra and more sequence patterns define the multivalency of those peptides and confers an increased cationic charge as well as hydrophobic groups. The steric hindrance given by the bulk, firstly, limit the access to the proteolytic site [35][36] and, secondly, seems to improve their activity by increasing the local concentration of peptide units with membranolytic activity [37]. Peptide structure is a pivotal point for the interaction with the membranes: the cationic charge allows the initial binding to a negatively charged layer; afterwards, while amphipathicity is necessary for membrane perturbation and peptide uptake, the hydrophobic groups are responsible for the carving [38].

3. Antibacterial Peptides and Their Mechanism of Action

Many factors can influence membrane perturbation and disruption by AMPs, i.e., amino acids sequence, the lipid composition of the membrane, peptide concentration as well as differences in membrane composition between eukaryotic and bacterial cells allow the AMPs to distinguish a microbial target from the host. Bacterial membranes are negatively charged due to the presence of anionic phospholipids groups, e.g., phosphatidylglycerol, phosphatidylserine, while eukaryotic cells possess groups with a neutral charge, e.g., phosphatidylcholine and phosphatidylethanolamine [39]. Moreover, the presence of cholesterol, a common feature in eukaryotic cells, is able to interact with AMPs either neutralizing or reducing their activity or stabilizing the phospholipid bilayer [40].

In Gram-positive bacteria, AMPs have to cross first the cell wall composed of crosslinked peptidoglycan with lipoteichoic acid prior to reaching the membrane whereas in Gram-negative they face a coat of lipopolysaccharide (LPS) followed by a phospholipidic outer membrane and a less cross-linked peptidoglycan layer [41]. Electrostatic interactions between the cationic peptide and the negatively charged components, e.g., lipopolysaccharide in Gram-negative and teichoic acid in Gram-positive, are the first steps to contribute to bacterial membrane affinity [42]. However, while AMPs seem to traverse the peptidoglycan layer with ease and access to the cytoplasmic membrane of the Gram-positive, they need to disrupt or perturb both outer and cytoplasmic membrane in Gram-negatives. Impedance in crossing or permeabilization results in loss of antimicrobial activity (**Figure 1**a (A,B)) [43].

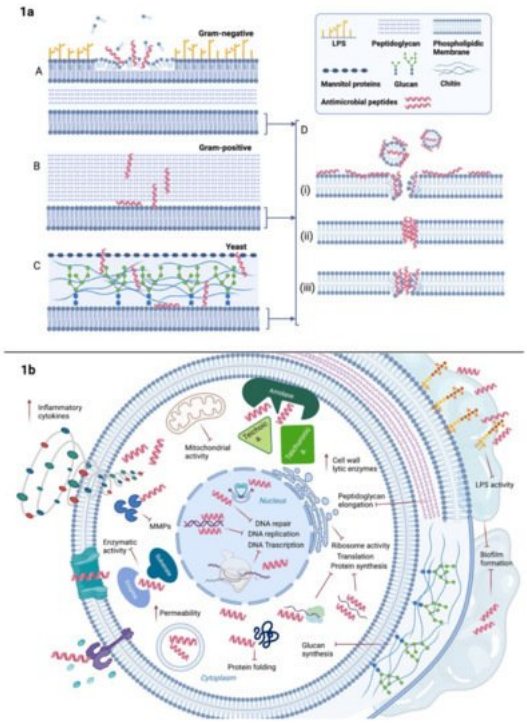

Figure 1. AMPs broad-spectrum antimicrobial activity. (**a**) Primarily, AMPs'action is based on their action on cytoplasmic membranes, i.e., perturbation or disruption. However, in presence of Gram-negative bacteria (**A**) AMPs have to firstly cross the outer phospholipidic membrane and secondly traverse the peptidoglycan layer before reaching the inner membrane. In Gram-positive bacteria (**B**) they navigate through the thick cell wall of peptidoglycan and in fungi (**C**), they encounter mannitol proteins, glucans and chitin prior to access to the cytoplasmic membrane. Once reached the phospholipidic bilayer, they induce perturbation via pore formation following either (**D**) (i) *carpet-like*, (ii) *barrel-stave* or (iii) or *toroidal pore* model depending on the peptide composition. (**b**) Besides pore formation, some AMPs bind some components and receptors on the extracellular side of the membrane, i.e., Toll-like receptors; others manage to enter the cytosol through direct penetration in vesicles or channels thus destabilizing the permeability and activating the inflammatory cytokines cascade. Intracellularly, they could also interfere with DNA or RNA leading to degradation and cell death. They may also affect mitochondrial activity or protein synthesis by targeting ribosome subunits or protein folding. In the case of bacterial cell wall, they can prevent elongation of peptidoglycan chains or hinder teichoic and teichuronic binding acids to amidases. Cell wall components inhibition will promote cell autolysis. In the extracellular space, AMPs can sequestrate LPS reducing the impact of endotoxins on the host's immune response. In fungal cells, AMPs can intervene on glucan synthesis thus blocking the building pieces of their wall. Further inhibitory action on biofilm matrix impairs the *quorum sensing* and improves the susceptibility of the single pathogens in both bacterial and fungal communities.

In order to explain the perturbation of the phospholipidic membranes operated by the AMPs, three main models have been proposed: *carpet-like*, *barrel-stave* and *toroidal pore* (**Figure 1**a (D)). Generally, when the ratio of peptide/lipids is low, AMPs interact with the phospholipidic layer of the membrane in a parallel manner, defined as *carpet-like* model, and interaction among the peptides or penetration in the hydrophobic core of the bilayer are not taking place [44]. Membrane integrity is disrupted and micelles are formed as in a detergent-like process [45]. With increasing AMPs ratio, they move to a perpendicular orientation until reaching such a concentration that they can cross the membrane forming pores (1:50–1:500 and more) [46][47]. A minimum length of ~22 amino acid for α-helix peptides is required to span the phospholipid layer, while β-sheet structures necessitate a minimum of 8 [48].

In the *barrel-stave*, interaction among peptides is a prerequisite as they mimic a transmembrane pore, whereas, in the case of the *toroidal* model, peptides are loosely arranged [49][50]. Despite the perturbation of the membrane seems to vary depending on the peptides, actually, the mechanisms of action are not completely well-defined and they are partially overlapping [51]. Moreover, all these models are based on the membrane perturbation but, then, the killing effect is not always enough to provide antimicrobial activity [52].

Besides membrane disruption, recent studies showed how peptides could act on other targets as well (**Figure 1**b) [53]. Some AMPs have shown their efficacy by binding some components and receptors on the extracellular side of the membrane and wall, thus destabilizing the permeability and/or activating intracellular signaling pathways that have, as a response, the inhibition or the activation of several functions.

The inhibitors of the nucleic acid biosynthesis seem to have a high binding affinity for both DNA and RNA because they share with nucleic acid-binding enzymes or substrates, homologous fragments of their sequences; an interesting example is represented by DNA-binding protein histone H2A [54]. Other mechanisms use the inhibition of the enzymes involved in the DNA/RNA biosynthesis, like DNA topoisomerase I preventing DNA relaxation [55], RNA polymerase blocking the

transcription [56] and gyrase impairing the supercoiling of DNA. [57] As a result, DNA/RNA degradation is induced and consequentially also cell death. There are several inhibitors of protein biosynthesis which alter the transcription and the translation but also the correct folding and the degradation of the protein. Usually, the AMPs that act on the protein biosynthesis target the ribosome subunits [58] but some others can interfere with the incorporation of histidine, uridine and thymidine [59][60], the amino acid synthesis pathways [61], the release factors on the ribosome [62], the regulation of sigma factors [63], the nucleotide and coenzyme transport [61] and the degradation of DNA-replication-associated proteins [64]. Some peptides influence protein folding, in particular, DnaK, the major Hsp70 of the chaperone pathway in *Escherichia coli*, which has been seen as an optimal target to prevent the refolding of misfolded proteins [65]. Another approach is linked to the inhibition of matrix metalloproteases, essential enzymes in microbial cell growth and homeostasis, i.e., serine protease, trypsin-like protease, elastase and chymotrypsins [66][67][68]. There are also inhibitors of cell division that block DNA replication or the mechanisms essential for the repair of DNA damages, then resulting in the block of the cell cycle, in the impairment of the chromosome separation, in the failure of septation, in the alteration of mitochondrial activity and in a substantial change in the cell morphology with clearly visible blebbing and elongation towards a filamentous shape [69][70].

Cell wall synthesis is another suitable target. Some AMPs act on lipid II by sequestrating it from the functional site [71][72] or by binding D-Ala-D-Ala residues of its precursor preventing the addition of *N*-acetylglucosamine and *N*-acetylmuramic acid in the structure, hence the peptidoglycan elongation [73].

4. AMPs—Goods vs. Bads, and the Long Way towards Clinical Application

There are obvious, multiple advantages of AMPs over classical antibiotics. AMPs are easy to synthesize, thanks to recent advances in automated protein synthesis, or can alternatively be produced in large quantities in heterologous expression systems, either in microbial cells or in plants [74]. In addition, AMPs are largely prone to chemical modification, aimed at overcoming inherent problems, such as susceptibility to enzymatic degradation, chemical/physical instability and toxicity to host cells, thus optimizing molecules' features and smoothing their pathway towards the clinics [75]. Broad-spectrum activity and rapid killing are other much-appreciated characteristics. Finally, AMPs are increasingly seen as a promising therapeutic alternative for treating biofilm-associated infections, one of the major threats in the field of bacterial infections [76].

A suitable instance of both the limitations to therapeutic use inherent to the nature itself of AMPs and the ways to overcome these is offered by the recent study of Wang Manchuriga and colleagues on temporins [77]. As many natural AMPs isolated from the skin of anuran amphibians (frogs and toads), temporins display a potent antimicrobial activity but this quality is often thwarted by elevated cytotoxicity, in particular against erythrocytes [78]. Working on temporin-GHa from *Hylarana guentheri*, Manchuriga and colleagues designed several analogs of the naturally-occurring sequence, modifying the type, position and number of charged residues. Some of the derived peptides displayed a significant reduction of hemolytic activity with respect to parent peptide while retaining potent antibacterial activity, but it was not possible to reduce cytotoxicity to zero without compromising antibacterial activity, confirming that a delicate balance of charge and other physico-chemical parameters (e.g., amphipathic and extension of hydrophobic surfaces) is necessary to obtain a plausible therapeutic lead [77].

One of the aspects that are often quoted in support of the (potential) use of AMPs in clinical practice is their low tendency to evoke antibiotic resistance. This tenet stems from the fact that AMPs generally (but not always, as specified above) hit the lipid component of the plasma membrane, a cellular component that is believed *per se* to be not easily modifiable in its basic physicochemical features by microbial targets. Although the slower emergence of resistance to AMPs with respect to conventional antibiotics is a reality, however, experience and much work have clearly shown that the reassuring thought that the complex phenomenon of resistance would not eventually thwart AMPs' value, is somewhat naïve and misleading. In fact, the long coevolution of microorganisms and AMPs has spurred the development of several resistance mechanisms. These include sequestration by bacterial enzymes, proteolytic degradation of peptides, efflux pumps to remove AMPs from the periplasmic space, alteration of components of bacterial surface to reduce surface attachment and permeability, down-regulation by immunomodulation [79][80][81][82].

Despite the limitations briefly outlined above, that have hampered their development in the classical drug discovery pipeline, AMPs are attracting continuous and ever-increasing interest as new antimicrobials agents. Out of some ~3000 molecules that have been isolated from different sources, just a handful have been the object of preclinical studies and further proceeded to clinical trials [82]. A recent analysis of AMPs patents from 2015 through 2020 has confirmed a long-standing trend, i.e., the fact that AMPs earmarked for clinical development are in vast majority analogs or derivatives of natural peptides, obtained through a template-based strategy aimed at enhancing the activity and stability of natural AMPs while reducing their toxicity [83].

Currently, just three AMPs have been approved by the U.S. Food and Drug Administration (FDA) for therapeutic use, i.e., gramicidin, colistin and daptomycin. Gramicidin has a long history. First isolated from *Bacillus brevis* over 70 years ago, gramicidin is active against a range of Gram-positive and Gram-negative bacteria, although its severe toxicity for human erythrocytes has a limited clinical indication to topical applications [84]. Polymyxin and colistin, which are cationic peptides in use for decades, have regained interest lately, due to their strong activity against multi-drug resistant Gram-negative

pathogens. Their ability to bind the lipid A component of LPS makes them precious, the last resource weapons to fight septic shock, notwithstanding their known nephrotoxicity. Resistance has emerged, however, and is spreading at an alarming pace, putting the effectiveness of these valuable therapeutics at risk [85][86]. Last but not least, daptomycin. This membrane-active cyclic lipopeptide has received the green light from the FDA in 2003 to treat Gram-positive infections. It is believed that its mechanism of action differs from that of other AMPs since daptomycin causes bacterial membrane depolarization rather than membrane disruption and pore formation [87]. In recent years, resistance in *Staphylococcus aureus* has been more and more frequently reported, and the search for substitutes that might prolong the clinical use of this important antibiotic is actively underway [88].

5. Conclusions

The challenging research for new antimicrobial entities is still ongoing but not without difficulties. New species of bacteria, fungi and viruses are emerging, and the most alarming fact is their intrinsic and sometimes multi-drug resistance to first-line drugs. These aspects together with the fast and global spread of resistance through horizontal transfer represent a serious threat for global health. An innovative approach involves the use of compounds inspired by nature and subsequently optimized to reach suitable features, i.e., low toxicity and strong activity. The result of this process is represented by synthetic peptides. Their broad mechanisms of action and the unlikely resistance that they generate, are important advantages and perhaps the key point for a shift towards new antimicrobial synthetic peptides-based treatments for the near future.

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