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Review Article

A guided tour through α-helical peptide antibiotics and their targets

Nils Preußke[1], Frank D. Sönnichsen[1] and Matthias Leippe[2]

[1]Otto Diels Institute for Organic Chemistry, Christian-Albrechts-Universität zu Kiel, 24118 Kiel, Germany; [2]Comparative Immunobiology, Zoological Institute, Christian-Albrechts-Universität zu Kiel, 24118 Kiel, Germany

Correspondence: Matthias Leippe (mleippe@zoologie.uni-kiel.de)

Nowadays, not only biologists, but also researchers from other disciplines such as chemistry, pharmacy, material sciences, or physics are working with antimicrobial peptides. This review is written for researchers and students working in or interested in the field of antimicrobial peptides—and especially those who do not have a profound biological background. To lay the ground for a thorough discussion on how AMPs act on cells, the architectures of mammalian and bacterial cell envelopes are described in detail because they are important targets of AMPs and provide the basis for their selectivity. The modes of action of α-helical AMPs (αAMPs) are not limited to different models of membrane permeabilization, but also include the disruption of intracellular processes, as well as the formation of fibrillary structures and their potential implications for antimicrobial activity. As biofilm-related infections are very difficult to treat with conventional antibiotics, they pose a major problem in the clinic. Therefore, this review also discusses the biological background of biofilm infections and the mode of actions of αAMPs against biofilms. The last chapter focusses on the design of αAMPs by providing an overview of historic milestones in αAMP design. It describes how modern αAMP design is aiming to produce peptides suitable to be applied in the clinic. Hence, the article concludes with a section on translational research discussing the prospects of αAMPs and remaining challenges on their way into the clinic.

Introduction

Microbial resistances against antibiotics are occurring worldwide at an increasing pace. This development poses a serious threat to the therapeutic efficiency of various medical treatments [1]. Since the discovery of penicillin by Sir Alexander Fleming in 1928, antibiotics have revolutionized modern medicine and are now indispensable for the treatment of bacterial infections and chronic diseases, for chemotherapies and for complex surgeries [1]. Microbial resistances against penicillin have been known even before it was available for civic use in 1943. In the 1950s, microbial resistance to antibiotics became a clinical problem that was efficiently overcome by developing new antibiotics until the late 1980s. Since the 1990s, multiresistant pathogens have developed leading to a surge of life threatening bacterial infections [1,2]. Two general causes result in the development of resistances in microbes. The first is the clinical and agricultural mis- or overuse of antibiotics that lead to evolutionary pressure among pathogens giving rise to resistances [1]. As a result, antibiotics are losing their therapeutic effect. The second reason is that less new antibiotics are being developed [1]. Resistances against existing antibiotics are unproblematic as long as new, potent drugs can replace compounds that have become ineffective. Since the 1980s, however, the number of approvals of new antibiotics is declining, while antibiotic resistant pathogens are on the rise threatening our society [1].

Mis- and overuse of antibiotics not only pose a threat to our health but also to the environment [3–5]. Unfortunately, the persistence of antibiotics, especially of fluoroquinolones, in the environment is very high due to their limited biodegradability [6]. For the potency of an antibiotic, chemical stability is an

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advantage because it contributes to a high bioavailability when no degradation of the compound occurs in the host. Once the substance is excreted, however, its stability is a major disadvantage if it precludes quantitative degradation and inactivation in the nature. Antibiotics used in veterinary medicine, as well as in animal husbandry find their way into the environment for example through the application of manure to fields to fertilize soils [7–10]. Although the use of antibiotics as growth promoters in livestock farming and aquaculture is banned in the European Union (EU) and many other countries, the global consumption of antibiotics in animal husbandry remains high and is predicted to increase on all continents [11]. As a direct consequence, the emergence of antibiotic resistant bacteria in animals bred in low- and middle-income countries is increasing [12].

High concentrations of antibiotics can also be found in municipal wastewater, especially in the effluents of hospitals. Modern sewage treatment is able to drastically reduce the amount of antibiotics [5,13] and the residual antibiotics in the effluents of a sewage treatment plant were shown not to drive the evolution of antibiotic-resistant bacteria [14,15]. However, heavy rains may lead to sewer overflows resulting in pollution of the environment with antibiotics or antibiotic resistant bacteria [15]. Furthermore, *in vitro* studies have shown that concentrations as low as 0.1 $\mu g\, l^{-1}$ of ciprofloxacin, a fluoroquinolone antibiotic, can induce resistance in bacteria [16]. This concentration is well below its concentrations found in some surface waters or in the effluents of sewage treatment plants [17]. Environmental pollution with antibiotics may change the microbiome of ecosystems and consequently affect the whole biotope [4]. Thus, it may lead to a qualitative and quantitative change in the bacterial fauna resulting for instance in altered decomposition rates of organic matter [3,4]. Additionally, toxic effects on the living fauna and the aforementioned increased emergence of bacterial resistances are plausible [4].

To combat the resistance problem, a drastic reduction of the overuse of antibiotics is necessary. This can mitigate the evolutionary pressure on non-resistant bacteria and slow down the emergence of new resistances. In addition, new antibiotics have to be developed to replace those that have become ineffective. Antimicrobial peptides (AMPs) are a class of peptides with antibiotic activities against a broad range of pathogens including bacteria, fungi, protozoa, or enveloped viruses. Especially the sub-class of α-helical AMPs (αAMPs) has been in the focus to develop new peptide-based antibiotics for many years.

The multifunctional, fast, and microbicidal immune response of the innate immune system is in part affected by gene-encoded host defense peptides (HDPs) that are present in virtually every eukaryotic organism [18–22]. Historically, the activity of those HDPs was entirely attributed to their direct inhibition or killing of pathogens [20], hence the original denomination as antimicrobial peptides (AMPs). Nowadays, both terms are often used synonymously. In this work, however, the term HDP will be used to describe peptides released by an organism to defend itself against infection. The mode of action of HDPs may therefore be immunomodulatory and/or directly antimicrobial [20,23]. Immunomodulatory peptides of synthetic origin will be termed innate defense regulators (IDRs) [23], while the term AMP includes synthetic and natural peptides with direct antimicrobial activity, i.e. killing or growth inhibition of bacteria evidenced *in vitro* or *in vivo*. By those definitions, AMP and HDP are no synonyms, although many peptides can be assigned to both groups. The immunomodulatory effects of HDPs have been reviewed extensively [19,20,23–25] and are beyond the scope of this review.

There is a broad sequential and structural variety among AMPs. Based on their secondary structure, AMPs can be divided into four groups: α-helical, β-sheet, extended, and loop AMPs [26], the largest groups being α-helical and β-sheet peptides [27]. Common features involve a positive net charge, allowing them to interact with the negatively charged microbial cell surface, and amphipathicity to insert into the lipid membrane [27,28]. Perturbation and disruption of the bacterial membrane is a common reason for the antimicrobial activity of AMPs, but they may also attack intracellular targets—often after 'self-promoted uptake' [26] across the bacterial membranes. For instance, some AMPs have been shown to bind deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) which may result in inhibition of RNA synthesis or protein synthesis, respectively. Regardless of the precise mode of action, knowledge about the cell envelope of mammalian and bacterial cells is crucial to understanding antimicrobial peptides.

Biological background

Once AMPs encounter a cell, they interact with the cell envelope. This interaction is dependent on both, the AMP and the architecture of the cell envelope. The term cell envelope includes the plasma membrane (also referred to as cytoplasmic membrane) and any of the following components if present: the periplasm, the peptidoglycan, the outer membrane, the lipopolysaccharide (LPS) layer, the surface layer (S-layer), and the glycocalyx.

In general, plasma membranes of bacteria and mammals are composed of lipids and contain membrane spanning integral proteins as well as peripheral proteins [29–31]. The current understanding of cell membranes follows the fluid mosaic model [29] which has been refined to include a higher complexity and organization, limited lateral

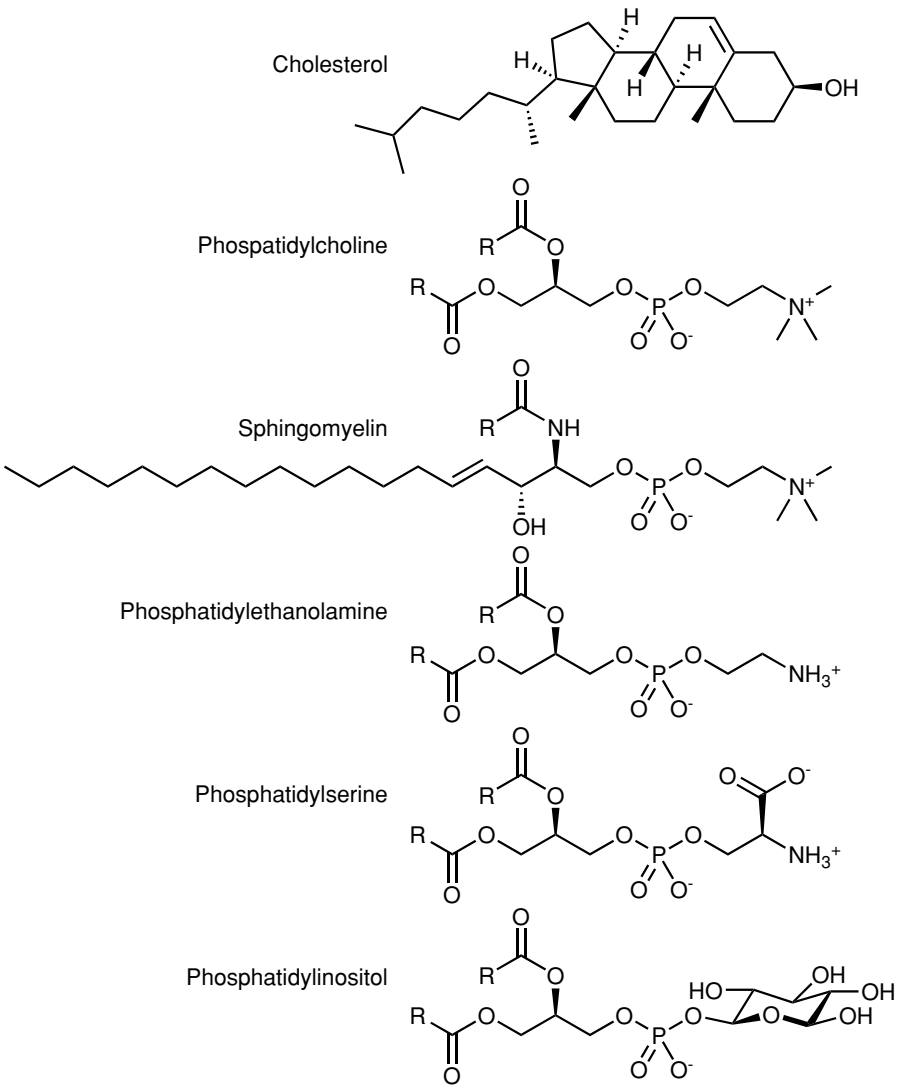

Figure 1. Chemical structures of lipids found in eukaryotic cell membranes in the order of their abundance (top = most abundant) [35]

The residues R are not necessarily identical and may be saturated or partially unsaturated linear aliphatic chains.

diffusion of lipids and proteins, as well as the existence of lipid domains, e.g. lipid rafts [30]. The composition of the plasma membrane differs between mammals and bacteria, providing a basis for AMP selectivity. However, there are also differences in mammalian membrane composition between species, tissues, and cell types [32]. Further, there is diversity in bacterial membrane composition, which not only depends on Gram-staining sensitivity or on the species, but also on the environment. Bacterial membranes are known to adapt to environmental challenges by changing the lipid composition [33]. This kind of diversity is not only present in the membrane composition, but also in other components of the cell envelope [34]. However, to design a broadly potent αAMP it is necessary to use a less diverse and more general model of the cell envelope that displays the major differences between mammalian and bacterial cells.

Mammalian cell envelopes

Mammalian cells have a relatively rigid plasma membrane as it has to compensate for the lack of a cell wall [35]. To achieve this high stability, equimolar quantities of rigid cholesterol and more flexible phospholipids (Figure 1) are incorporated into the mammalian plasma membrane [35]. The most abundant phospholipids in the membrane are zwitterionic, i.e. phosphatidylcholine, sphingomyelin, and phosphatidylethanolamine [35]. Lipids with a net negative

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charge such as phosphatidylinositol and phosphatidylserine only make up circa 10 mol% of all lipid compounds in the mammalian plasma membrane [35]. The distribution of phospholipids differs between its inner leaflet and outer leaflet [35–40]. Phosphatidylserine [36] and phosphatidylethanolamine are mainly found in the cytosolic leaflet, whereas the outer leaflet is dominated by sphingomyelin and phosphatidylcholine [37–40]. As a result, the plasma membrane of eukaryotic cells displays an overall neutral charge to the exterior of the cell.

The two leaflets of the mammalian cytoplasmic membrane are not only different regarding the distribution of the lipid head groups but also in terms of acyl chain unsaturation [40]. The exoplasmic leaflet is more ordered and less fluid because its lipids have a rather low degree of unsaturated acyl chains, whereas the lipids of the inner leaflet have a higher degree of unsaturation rendering the inner leaflet more diffusive [40]. In addition to the lipid fraction, eukaryotic plasma membranes are densely populated by proteins [41]. A typical lipid–protein ratio by weight of roughly 1:1 can be found in most eukaryotic plasma membranes [31]. However, lipids and proteins are not evenly distributed in the membrane but tend to form domains of specifically associated lipids and proteins [40,41].

Due to the lipophilic nature of the lipid bilayer, the cytoplasmic membranes constitute a barrier for hydrophilic ions. This allows the build-up of an electrostatic gradient through the membrane, the so-called transmembrane potential. Ion pumps in the mammalian plasma membrane generate a high K^+ concentration and a low Na^+ concentration inside the cell [42]. Especially the Na^+/K^+-ATPase is involved in the generation of these concentration gradients. It transports three sodium ions from the cytoplasm to the extracellular medium in exchange for the import of only two potassium ions resulting in the build-up of a negative transmembrane potential [43,44]. The potential is increased (i.e. more negative) by continuous conductance of K^+ along its concentration gradient through selective K^+ channels [42]. In consequence, the resulting transmembrane potential ($U_{eq} \approx$ -70 mV) [42,45] is close to the Nernstian equilibrium potential of K^+ [45,46].

On the outside of the plasma membrane lies the glycocalyx [47], which is particularly well studied in endothelial cells [48]. Covering every living cell in nature [47], the glycocalyx is a mesh of glycans, which are the carbohydrate portions of proteoglycans, glycoproteins, and glycolipids. In mammals, the glycocalyx is anchored to the plasma membrane via lipids or membrane proteins. The glycans usually are large, flexible, highly hydrated, and often anionic [49]. Because these glycans are displayed at the cell surface, they constitute a major interface for the interaction with neighboring cells, symbionts, pathogens, and the environment in general [47,49].

When assessing the activity of AMPs, their effects on bacteria as well as on mammalian cells are usually tested. While common bacteria such as *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Staphylococcus aureus* (*S. aureus*) are easily grown in liquid and on solid media, culturing primary cells derived from healthy tissues is very complex. Therefore, mammalian cell cultures mainly rely on tumor-derived cell lines [50]. Unfortunately, tumor cells have a different membrane composition than healthy cells [51]. In consequence, erythrocytes (also referred to as red blood cells, RBCs) are often used in studies to represent mammalian cells [52]. They can be easily obtained through blood donations and their overall membrane composition and lipid distribution between the inner and outer leaflet is in fact similar to the prototypical mammalian cell envelope described above [53,54]. Another advantage of erythrocytes is the simple detectability of hemoglobin leakage upon membrane disruption by an AMP, which is exploited in so-called hemolysis assays [55,56]. These features qualify RBCs to represent mammalian cells in hemolysis assays as a first indicator of AMP toxicity. However, there are certain discrepancies between erythrocytes and other mammalian cell types, that limit the applicability of RBCs as a general model of mammalian cells. For instance, the transmembrane potential of human RBCs is with approximately $U_{eq} \approx -10$ mV [57–59] less negative than that of other mammalian cells. Presumably, this makes human erythrocytes more resistant against cationic AMPs because larger, i.e. more negative, transmembrane potentials facilitate the membrane disruption through cationic AMPs. Secondly, the RBC membrane is stabilized by a 2D network of skeletal protein filaments, which is located inside the cell close to the membrane [54]. The membrane skeleton is covalently attached to transmembrane proteins and non-covalently bound by anionic phospholipids of the inner leaflet [54]. It confers the necessary stability and elasticity to the erythrocyte membrane enabling drastic deformations of RBCs [54]. It appears plausible that this stabilization not only prevents membrane breakup, vesiculation, or cell fragmentation under mechanical stress but also increases the resistance against cationic AMPs. A further drawback of using mammalian RBCs to assess the toxicity of AMPs to mammalian cells is their lack of a nucleus and cell organelles [53]. Thus, hemolysis assays provide no or only limited insights how an AMP may affect internal targets of mammalian cells.

In conclusion, the absence of hemolytic behavior in AMPs does not necessarily imply low toxicity towards mammalian cells as hemolysis assays tend to underestimate the lytic potential of AMPs and do not report on how internal targets would be affected. Nonetheless, erythrocytes are well suited to serve as an inexpensive and easily obtainable model for the mammalian cell membrane and hemolysis assays may provide first insights into the lytic behavior of AMPs.

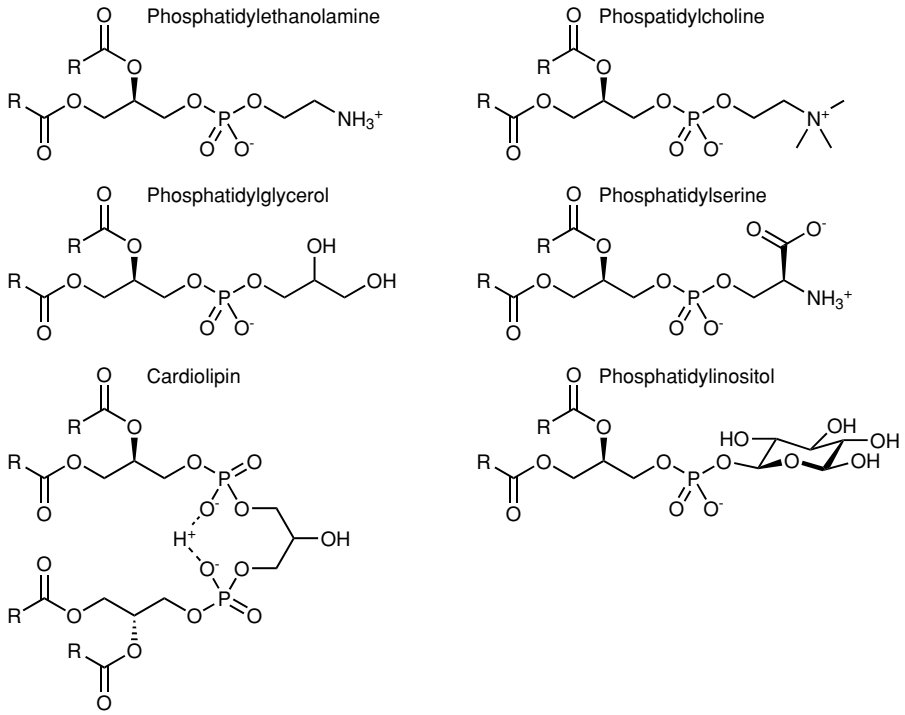

Figure 2. Chemical structures of lipid head groups found in bacterial cell membranes

Their abundance is highly dependent on the bacterial species as well as environmental factors. The residues R are not necessarily identical and may be saturated or partially unsaturated linear aliphatic chains [61].

Bacterial cell envelopes

Like eukaryotes, bacteria possess a lipid bilayer membrane bordering the cytoplasm [60], albeit with a different lipid composition. Main components of the bacterial cytoplasmic membrane are the zwitterionic phosphatidylethanolamine, as well as the anionic lipids phosphatidylglycerol and cardiolipin [61] (Figure 2). Additionally, bacteria may incorporate phosphatidylcholine, phosphatidylserine, phosphatidylinositol or phosphate-lacking lipids into their membrane [33,34,61]. As a result of the lipid composition, the surface of the bacterial plasma membrane is negatively charged, which is in contrast to the overall neutral outer leaflet of the mammalian cytoplasmic membrane. Bacteria lack intracellular organelles and must incorporate many membrane proteins into the cytoplasmic membrane, the eukaryotic equivalents of which are associated with membranes of organelles [34]. For instance, in eukaryotes the complexes of the respiratory chain are found in the inner membranes of mitochondria, where they produce a proton gradient and *ipso facto* an electrostatic gradient between the mitochondrial matrix (high pH) and the intermembrane space (low pH) [62]. This proton gradient is exploited by ATP synthase that uses the proton motive force to synthesize adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and phosphate. In respiring bacteria, these functions are located in the cytoplasmic membrane pumping protons out of the bacterium [60]. This leads to a larger transmembrane potential compared to eukaryotic cells. Typical transmembrane potentials of respiring, neutrophilic bacteria reach levels around $U_{eq} \approx -150$ mV [63].

Apart from these differences in membrane composition and transmembrane potential, the cell envelope of bacteria differs greatly from that of mammals because bacteria generally possess a cell wall (Figure 3). Most bacteria can be divided into being either Gram-positive or Gram-negative, depending on their sensitivity to the Gram-staining method [60]. Typical representatives of Gram-positive bacteria and well-studied model organisms are *Micrococcus luteus* (*M. luteus*), *Bacillus subtilis* (*B. subtilis*), and *S. aureus* [34]. Gram-positive bacteria mainly incorporate phosphatidylglycerol, cardiolipin, and the lipid part of lipoteichoic acids into their plasma membrane [61]. Outside the cytoplasmic membrane lies the cell wall composed of its main component peptidoglycan as well as teichoic acids [34,60]. Peptidoglycan is composed of alternating, β-(1,4)-linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) [60]. The long strands are interconnected by short, overall negatively charged peptide chains containing

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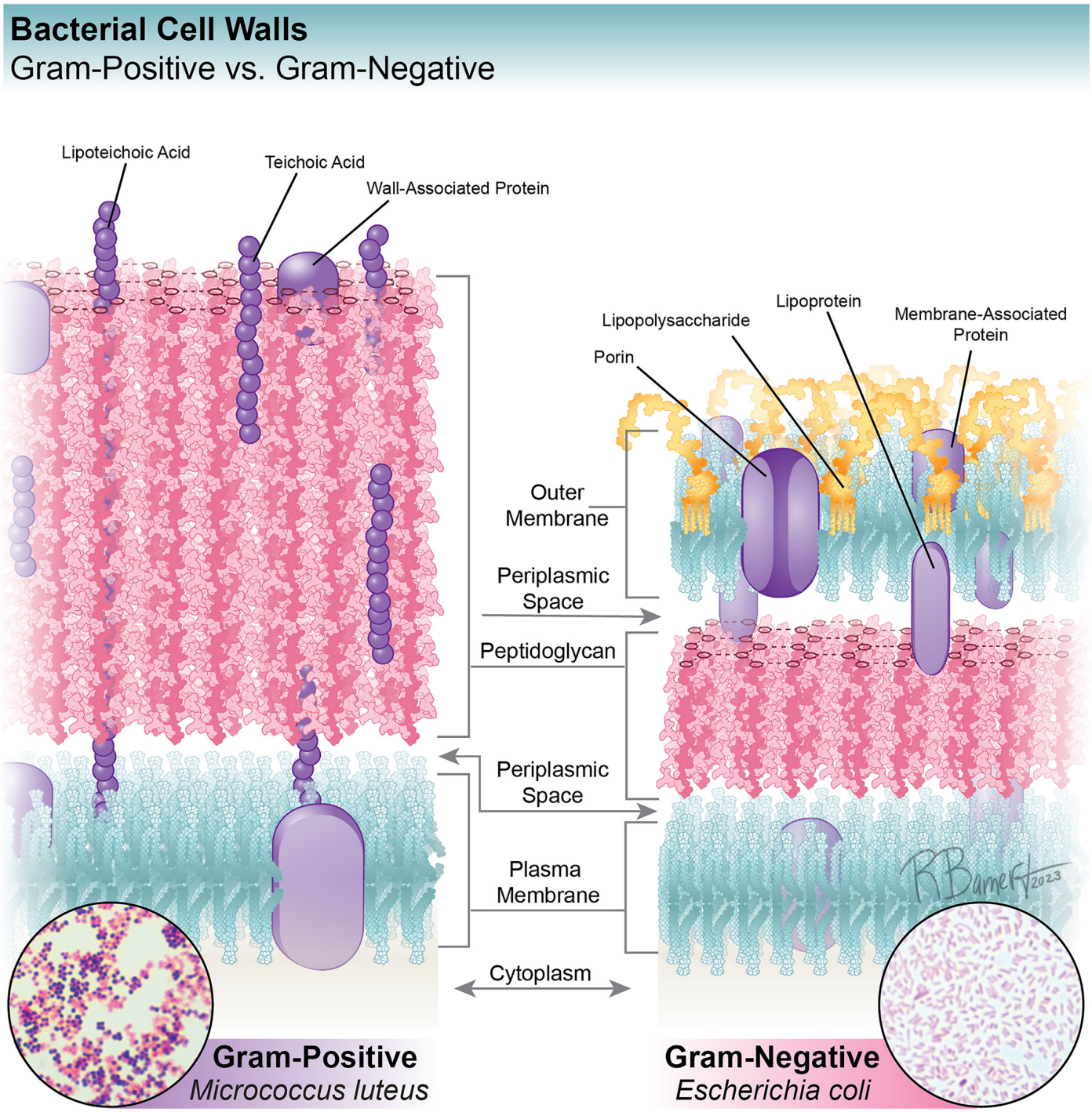

Figure 3. Cell wall and membrane(s) of Gram-positive and Gram-negative bacteria

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proteinogenic and non-proteinogenic amino acids [60]. Usually, the cell wall of Gram-positive bacteria is thick (approximately 30–100 nm) and contains multiple layers of peptidoglycan [60,61]. Embedded in the peptidoglycan are teichoic acids, i.e. linear polymers of glycerol phosphate, glucosyl phosphate or ribitol phosphate [34,60]. There are D-alanine or D-glucose molecules attached to the glycerol or ribitol moieties of the teichoic acids [60]. The strands

of wall teichoic acids are covalently bound to the peptidoglycan, whereas lipoteichoic acids are connected to membrane lipids [34,60]. Due to their negative charge, teichoic acids bind divalent cations such as Ca^{2+} and Mg^{2+} [34,60]. Strands of wall teichoic acids are known to protrude through and beyond the peptidoglycan layers into the aqueous environment of the cell [34].

The cell envelope of Gram-negative bacteria includes at least the cytoplasmic membrane, the periplasm, a thin peptidoglycan layer, a second membrane termed the outer membrane and the LPS layer [60]. Most research on Gram-negative bacteria targeted *E. coli* [33,34,64], a common model organism in microbiology. Like the plasma membrane of Gram-positive bacteria, that of Gram-negative bacteria is mainly composed of lipids and embedded proteins. The lipid fraction typically contains phosphatidylethanolamine and phosphatidylglycerol, usually with higher amounts of phosphatidylethanolamine [61]. Additionally, cardiolipin is abundant with a typical share of 5–10% of all lipids [61]. Phosphatidylserine and other lipids are less common [34]. Outside of the cytoplasmic membrane lies the periplasm which is isolated from the exterior of the cell by the outer membrane [34]. The periplasm is more viscous than the cytoplasm due to a high content of proteins and solutes [34,60]. Embedded into the periplasm with a gap to both neighboring membranes lies a thin layer of overall negatively charged peptidoglycan (approximately 3–5 nm) [60,61]. As opposed to the Gram-positive cell wall it contains no teichoic acids. The peptidoglycan is covalently attached to the inner leaflet of the outer membrane by the Braun lipoprotein, also termed Lpp [34,60]. The outer membrane differs significantly between its inner and outer leaflet. Whereas the inner leaflet of the outer membrane displays a similar lipid composition as the cytoplasmic membrane [61], the outer leaflet of the outer membrane is mainly composed of lipopolysaccharides (LPS) [34]. Although there is great variability in the components of LPS among different Gram-negative bacterial species, its overall structure is well preserved [60]. The lipid part of LPS is lipid A, a phospholipid comprising a doubly phosphorylated glucosamine disaccharide head group equipped with six to seven acyl chains [34,60]. Attached to lipid A there is a branched and partly phosphorylated oligosaccharide core. Finally, the core is connected to the O-specific polysaccharide, also referred to as O-antigen, a long, highly variable polysaccharide strand [60]. The protein fraction of the outer membrane includes many lipoproteins of unknown function attached to the inner leaflet [34]. Further it contains porins and other transmembrane proteins that allow diffusion of small molecules across the outer membrane [34]. Proteins, however, cannot pass these porins and remain located within the periplasm [60]. The outer membrane contains only few embedded enzymes, but one of them, OmpT, is of particular importance. The active site of this protease is accessible from outside the cell. It is able to degrade peptides and may therefore confer resistance against AMPs. Accordingly, it may facilitate the pathogenic colonialization of the human intestine by enterohemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC) [65].

Some, but not all Gram-negative and Gram-positive bacteria possess a surface layer (S-layer) [66]. The bacterial S-layer is a paracrystalline monolayer of (glyco)proteins, typically 5–10 nm thick and generally composed of a single molecular species [66]. The S-layer forms a sheath covering the entire bacterium. The monomers are arranged in a defined, 2D lattice. In Gram-positive bacteria the S-layer is non-covalently attached to secondary cell wall polymers which in turn are covalently bound to the peptidoglycan [67], whereas in Gram-negative bacteria the S-layer adheres to the glycan of LPS [66]. S-layers typically possess regularly distributed, uniform pores between 2 and 8 nm in diameter [66], which is large enough for the passage of peptides and small proteins. Despite these pores, the S-layer may provide enhanced resistance against AMPs in bacteria, possibly through electrostatic repulsion of the positively charged AMPs [68].

The outmost layer of each cell is the typically anionic and highly hydrated glycocalyx [69]. The main components of the glycocalyx are polysaccharides and (glyco)proteins [69]. For instance, teichoic acids, glycolipids, glycans of LPS, or glycans of S-layers belong to the glycocalyx if they protrude into the extracellular medium. There are varying definitions of the glycocalyx [60,69] and the simplest [60] distinguishes two categories. The term 'capsule' describes a dense, definite network of biopolymers that is firmly bound to the cell envelope [60]. A slime layer, in contrast, is less dense and easily deformed. It is rather loosely attached and may at least partly shed into the extracellular space [60,69].

Selectivity of AMPs

The different cell envelopes of bacterial and mammalian cells allow for AMPs to display selectivity, i.e. to have a bactericidal or bacteriostatic effect while mammalian host cells are left unaffected. The ratio of minimal hemolytic concentration (MHC) and minimal inhibitory concentration (MIC, i.e. the concentration of a substance that is sufficient to inhibit bacterial proliferation) is referred to as therapeutic index (TI) and is commonly used as a measure for the selectivity of AMPs [55]. A higher therapeutic index indicates higher activity towards bacteria than towards

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erythrocytes. Three major differences between the plasma membrane of bacteria and mammals are mainly responsible for the preference of AMPs to target bacteria: the presence of anionic lipids at the bacterial outer leaflet, the absence of cholesterol in the bacterial membrane, and the increased (i.e. more negative) electrostatic potential across the bacterial plasma membrane.

Anionic lipids are abundant in the outer leaflet of the bacterial plasma membrane but scarce in the outer leaflet of mammalian plasma membranes. In consequence, cationic AMPs often show higher association constants with, and more efficient membrane permeabilization of bacterial membranes compared to mammalian ones [70]. According to the currently accepted modes of action (described in detail in Chapter 3. Mode of action), the first interaction between cationic AMPs and the envelope of any cell is mediated by electrostatics. Attracted by charge–charge interactions, cationic AMPs are able to diffuse into the negatively charged glycocalyx. In bacteria, a diffusion deeper into the cell envelope is facilitated, because not only the glycocalyx, but also LPS (in Gram-negative bacteria), peptidoglycan, and the plasma membrane display negative charges and diffusion of the AMPs to the cytoplasmic membrane proceeds primarily along its concentration gradient. In mammalian cells, however, the cytoplasmic membrane beneath the glycocalyx is neutral on the outside. Association with the membrane is, therefore, hampered by attractive forces between the glycocalyx and the peptides [71].

The second major difference between bacterial and mammalian plasma membranes is the presence of cholesterol. With equimolar quantities of cholesterol and phospholipids, cholesterol is highly abundant in the mammalian plasma membrane [35]. Due to its rigid backbone, the presence of cholesterol has an ordering effect on the acyl chains of neighboring phospholipids leading to reduced density fluctuations, overall thickening, and decreased permeability of the membrane [72]. Nonetheless, some liquid properties of the membrane, such as translational disorder and lateral diffusion of the lipids, are maintained in this cholesterol-induced, so-called liquid-ordered state [72]. Cholesterol has a higher affinity for sphingomyelin or other phospholipids with saturated hydrocarbon tails than for unsaturated lipids which can result in the formation of two distinct phases referred to as membrane domains [73–75]. Studies of model membranes showed that cholesterol influences the formation of lipid domains or lipid rafts, i.e. lateral structures with different membrane constituents (phospholipids, cholesterol, and peripheral or integral proteins) [72–75]. The highly stable liquid-ordered phase containing cholesterol and saturated lipids is relatively resistant to detergents [73] or AMPs [74,75], whereas domains in a liquid-disordered state containing unsaturated phospholipids and low to no amounts of cholesterol are not [73–75]. Some studies argue that cholesterol might have only a limited effect on the resistance of mammalian cells against AMPs, because domains exist that contain few or no cholesterol and which are therefore susceptible to disruption by antimicrobial peptides [74,75]. This interpretation is contrasted by a study assessing the effect of granulysin, a cationic pore-forming protein of 74 residues with a mode of action comparable to AMPs, on model membranes of different compositions [76]. Membranes composed of lipid extracts from erythrocytes or different lipid mixtures containing cholesterol were completely resistant to pore formation by granulysin, whereas membranes lacking cholesterol were highly susceptible. It should be noted, that domain formation was not observed in these membranes, but only occurred upon heating the membranes above 50 °C [76]. Cholesterol was also found to not only stabilize artificial membranes containing saturated lipids, but also those comprising mainly unsaturated phospholipids [75,77]. It appears plausible that the high concentration of cholesterol in eukaryotic plasma membranes results in the formation of lipid rafts with high concentrations of cholesterol and very high resistance to AMPs. A fraction of the cholesterol, however, may diffuse along its concentration gradient into domains with lower cholesterol affinity and higher susceptibility to AMPs. In those more vulnerable domains it may significantly improve the resistance to AMPs and hence increase the overall stability of the cell.

In addition to the presence of anionic lipids and the absence of cholesterol, the efficiency of AMPs against bacteria is increased by the more negative bacterial transmembrane potential. The bacterial transmembrane potential ranges around $U_{eq} \approx -150$ mV [63], whereas the typical transmembrane potential of mammalian cells is about $U_{eq} \approx -70$ mV [42,45]. In a study with bacterial membrane-mimicking planar lipid bilayers, highly negative transmembrane potentials (−180 mV) were required to initiate AMP-induced conductance across the membrane, i.e. membrane permeabilization. Subsequent reduction of the voltage to −80 mV often resulted in reduced activity of the peptides [78].

The presented differences are general observations and exceptions do occur. Nonetheless, these three differences are key factors for the specificity of many AMPs against bacteria.

Infections involving bacterial biofilms

Bacterial infections can be classified as acute or chronic. While acute infections in immunocompetent individuals mainly involve planktonic, i.e. free-floating, bacteria, chronic infections often occur in immunocompromised patients

and involve biofilm formation [79]. Rodney M. Donlan and John William Costerton define a biofilm as 'a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription' [80]. Typically, biofilm-related bacterial infections are more difficult to treat and to cure than those involving planktonic bacteria of the same species. In consequence, effective antibiotics for the treatment of bacterial biofilm infections are scarce. Here, we discuss the biology of biofilm infections to lay the grounds for understanding how AMPs can be used to eradicate biofilms *in vivo*.

When exposed to rapidly flowing or turbulent conditions, planktonic bacteria tend to attach themselves to surfaces and to one another [80]. In patients, biofilms commonly form on inert surfaces like those of medical devices or on dead tissues [79]. Proliferation of the sessile bacteria results in the formation of so-called microcolonies and biofilm growth [60]. Adhesion to a solid surface triggers the expression of biofilm-specific genes [60,81] which leads to the production of extracellular polymeric substances (EPS) to form a matrix surrounding the bacteria [80]. While approximately 85% of a biofilm's volume is made up of the highly hydrated EPS matrix, living cells contribute only 15% [80]. Initially, the EPS matrix is mainly composed of anionic polysaccharides excreted by bacteria, e.g. alginate [81,82], but in later stages it may also contain proteins and nucleic acids released from dead cells [60]. During biofilm growth, bacteria of other species may attach to the EPS of a microcolony resulting in a heterogeneous biofilm [60,79]. Originally, biofilms found *in vivo* were thought to have the same architecture as biofilms grown *in vitro* [80], which have a mushroom-like structure with water channels facilitating convective flow [83] to transport nutrients from the environment into the biofilm [60,80]. However, biofilms commonly found at sites of infection tend to be smaller and do not form mushroom-like structures [84]. Presumably, the presence of antibiotics and the immune response of the host limit the size of biofilm patches in patients [84]. Nonetheless, ecological niches with different levels of nutrients, oxygen, and other environmental factors exist inside the biofilm that benefit different bacterial phenotypes or species [60,82]. Lack of nutrients and oxygen deep inside the biofilm may force bacteria into a persister state with an extremely slowed metabolism [80]. Biofilm-associated cells may communicate via quorum sensing to coordinate biofilm growth or dispersal [60,85]. Dispersal may be achieved actively upon the detection of a quorum signal, or it may be a result of mechanical force breaking pieces off the biofilm [80].

It has been known for more than 20 years that biofilms are responsible for many chronic infections [79]. The biofilm-associated cells are not overtly pathogenic, because bacteria and produced toxins are trapped inside the EPS matrix or neutralized by antibodies at the biofilm surface [86]. However, concerted release of highly pathogenic planktonic bacteria upon quorum sensing-initiated biofilm dispersal may overwhelm the host's immune system leading to symptoms of acute infection [79]. These may be treated with conventional antibiotics that kill the released planktonic bacteria [79]. Unfortunately, some cells within the biofilm are able to resist the antibiotics and are recalcitrant to the host immune system. Thus, symptoms may keep recurring in patients with chronic biofilm infections even after multiple periods of treatment [79].

Adaptation to the life in a biofilm significantly protects sessile bacteria from environmental influences. Although biofilm growth induces antibody production in the host, macrophages fail to engulf the cells within the EPS-protected microcolonies [79]. Further, the EPS matrix may—depending on the type of antibiotic and the biofilm—slow the diffusion of antibiotics [87]. In case of a significantly slowed diffusion, bacteria may have sufficient time to adapt to the slowly increasing antibiotic concentrations [87]. In general, a reduced growth rate due to a lack of nutrients may increase the tolerance of antibiotics in bacteria [88]. Sessile bacteria often display a slower metabolism compared to their planktonic counterparts. In consequence, antibiotics interfering with the bacterial metabolism act very slowly on biofilms [60], which adds to the recalcitrance provided by other protective aspects of the biofilm. For instance, adaptation to the life in a biofilm results in the expression of biofilm-related genes that increase stress tolerance [60], e.g. by increasing the activity of efflux pumps in sessile bacteria [87]. Of course, not all cells in a biofilm are effectively protected and most do not survive an antibiotic therapy. However, some persister cells may survive the treatment [79,87]. Once the antibiotic treatment has ended, these few persisting cells wake up from their inactive state and repopulate the biofilm, feeding on the plethora of nutrients released from the dead bacteria around them.

The recurring biofilm-related symptoms of acute infection may require repeated treatment of a patient with antibiotics. This further increases the risk of the development of inherent antibiotic resistance in the microbes. In addition, the biofilm environment favors horizontal gene transfer facilitating the rapid spread of resistance genes. Inherent antibiotic resistance and biofilm-mediated recalcitrance may have synergistic effects making biofilm infections even harder to treat [87].

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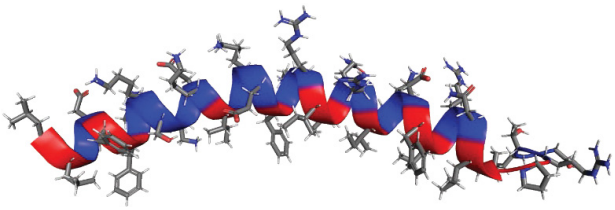
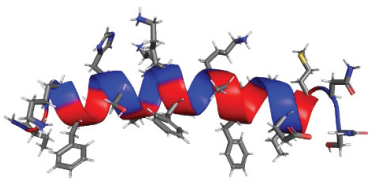


LL-37: LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES



Magainin 2: GIGKFLHSAKKFGKAFVGEIMNS–NH_2

Figure 4. Structure of two representative αAMPs, LL-37 (top) and magainin 2 (bottom), when bound to sodium dodecyl sulfate (SDS) or dodecylphosphocholine (DPC) micelles, respectively

Hydrophilic residues are marked by a blue ribbon, while for hydrophobic residues the ribbon is colored in red. The hydrophobic residues are buried in the detergent micelles (not shown) driving the formation of the amphipathic α-helix. The depiction of LL-37 is derived from PDB entry 2k6o (1/4). The depiction of magainin 2 is derived from PDB entry 2mag (1/10).

Mode of action

α-helical AMPs (αAMPs) are a large group of linear AMPs that display an α-helical structure in the membrane-associated state [27,28] (Figure 4). Sometimes, the entire molecule is helically structured when adsorbed to the membrane or a membrane mimic [89]. More often, however, kinks within a helix [90–93] or disordered termini [90,92–95] are observed. The helical conformation in a membrane environment is a result of the amphipathicity of αAMPs. Typically, these peptides present a hydrophilic and a hydrophobic side of roughly similar size when displayed in a helical wheel projection [28]. The helical conformation is assumed, when the burial of the hydrophobic side of the helix into the membrane compensates for the entropic costs of helix formation. In aqueous solution, however, αAMPs often are highly flexible with little or no secondary structure [27,28].

In vitro, αAMPs possess a high activity against many potential pathogens such as Gram-positive and Gram-negative bacteria, fungi, protozoa, or viruses [27,28,96]. This review focuses on the interactions of αAMPs with bacteria as those are well understood [26–28,97] and most important for this work. Early studies found αAMPs composed of only D-amino acids to have similar activities as their all-L-enantiomers, which precluded a receptor based mechanism [98,99]. Further investigations revealed two distinct modes of action by which αAMPs can act on bacteria [27,28,97]. One of them is membrane permeabilization, which includes procedures that weaken and disrupt the bacterial membrane(s). This will eventually lead to loss of transmembrane potential, nutrients, and other essential cytosolic constituents ultimately resulting in growth inhibition (bacteriostatic effect) or cell death (bactericidal effect) [27,28]. The other mode of action targets intracellular components and functions [27,28]. AMPs are able to reach the bacterial cytosol where they may associate with nucleic acids or proteins to block essential cellular processes. It is possible that one αAMP exerts both mechanisms, depending on its structure and concentration as well as membrane architecture [28]. Regardless of the mode of action, interaction with the cell envelope is crucial for the activity of αAMPs.

The interactions of cationic αAMPs with Gram-negative bacteria are well understood [26]. First, the initially disordered peptides diffuse into the anionic glycocalyx attracted by charge–charge interactions. If present, an overall neutral S-layer is usually no significant barrier, as αAMPs are small enough to pass the pores. The first interaction harmful to the bacterium occurs when αAMPs encounter the LPS layer of the outer membrane. Divalent metal ions confer rigidity to the cell envelope by non-covalently cross-linking the polysaccharide chains of the LPS layer [26]. Replacement of those metal ions by the much larger cationic αAMPs weakens the outer membrane and facilitates

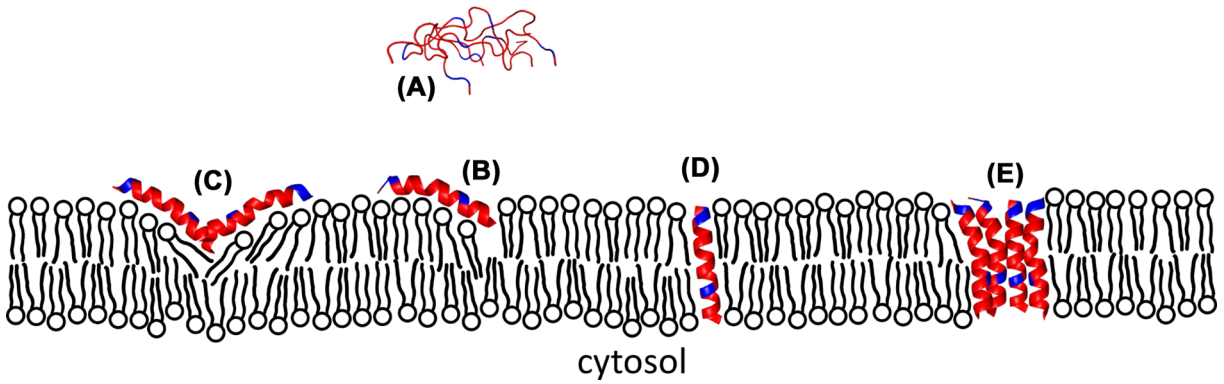


Figure 5. Barrel stave mechanism

Helical structures of alamethicin are taken from its crystal structure (pdb entry 1amt). Hydrophilic residues of the peptides are colored in blue, hydrophobic residues in red. The phospholipids of the membrane are represented by black circles (head groups) and two wavy black lines (hydrocarbon tails). Letters A to E describe critical steps in the temporal progression of the pore formation. (**A**) Alamethicin forms unstructured aggregates in solution. (**B**) Horizontal insertion into the membrane is accompanied by helix formation of the peptide. (**C**) Lateral diffusion leads to aggregation of the helices and more severe disturbance of the membrane. (**D**) Alternatively, monomeric peptides may first insert vertically into the membrane before they aggregate. (**E**) Formation of the barrel stave pore is a result of peptide self-assembly.

interaction with the lipid core of the outer membrane [26]. Interaction with the lipid A moiety of LPS results in helix formation of αAMPs due to the burial of the hydrophobic site in the lipid bilayer [100,101]. Additionally, some αAMPs may directly bind lipid A [101–104]. This interaction counteracts the endotoxic activity of released lipid A and further destabilizes the outer membrane [26]. Once sufficient AMPs are located at the outer membrane the formation of pores or even lysis of the outer membrane facilitates the uptake of more AMPs—a process aptly described as 'self-promoted uptake' [26]. After having passed the outer membrane, the peptides diffuse into the peptidoglycan layer until they reach the inner membrane.

With Gram-positive bacteria, diffusion into the glycocalyx and through the S-layer by αAMPs is a process similar to Gram-negative bacteria as these structures are alike in both bacterial types. The presence of negatively charged teichoic acids embedded in the peptidoglycan of Gram-positive bacteria facilitates the diffusion deep into the cell wall [28]. However, the thick peptidoglycan layer of Gram-positive bacteria may also sequester the cationic peptides thus reducing their antimicrobial activity and slowing their bactericidal effect [105].

After having reached the cytoplasmic membrane, the further processes are again similar between Gram-negative and Gram-positive bacteria. Attracted by the anionic phospholipids, αAMPs accumulate at the surface of the cytoplasmic membrane [28]. Hydrophobic residues partition into the lipophilic tail region of the outer leaflet of the membrane, while polar residues remain in the head group region. As a result, the peptide assumes an α-helical conformation while floating in the membrane at the interface to the periplasm. Its helical axis is oriented parallel to the membrane surface [28]. Once a critical concentration is reached, membrane permeabilization may proceed according to one of several proposed mechanisms.

Barrel stave mechanism

The barrel stave mechanism was the first model to explain the mode of action of pore-forming peptides. It was proposed in 1974 to explain the voltage-, lipid-, and concentration-dependent conductance of membranes in the presence of alamethicin [106]. Although the original model was based on an incorrect cyclic alamethicin structure [106], the general barrel stave mechanism was confirmed to apply to alamethicin in later studies [107,108]. Alamethicin is not cationic but highly hydrophobic and displays only one charged residue which is glutamic acid [109]. Because of its hydrophobicity, alamethicin is largely unstructured and forms aggregates in solution [110] (Figure 5A). The barrel stave mechanism is assumed to start with the accumulation of monomeric peptides in α-helical conformation at the membrane surface [27] (Figure 5B). Lateral diffusion leads to the formation of peptide aggregates stabilized by peptide–peptide interactions, locally disturbing the membrane order [27] (Figure 5C). This facilitates the insertion of the peptide aggregates into the membrane and the formation of the barrel stave pore [27]. An alternative mechanism of barrel stave pore formation was proposed in which monomeric alamethicin inserts vertically into the membrane

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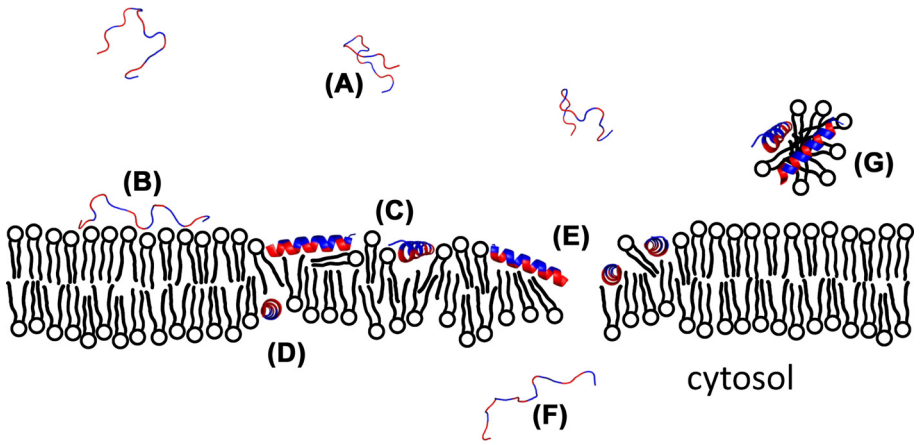

Figure 6. Carpet mechanism

Hydrophilic residues of the peptides are colored in blue, hydrophobic residues in red. The phospholipids of the membrane are represented by black circles (head groups) and two wavy black lines (hydrocarbon tails). Letters A to G describe critical steps in the temporal progression of the membrane disruption. (**A**) Cationic αAMPs are monomeric and unstructured in solution. (**B**) They are attracted to the membrane surface by charge-charge interactions. (**C**) Insertion into the membrane and formation of α-helical structure is driven by hydrophobic interactions. (**D**) Accumulation leads to weakening of the membrane facilitating diffusion to the inner leaflet. (**E**) Ruptures may spontaneously form in the membrane. (**F**) This facilitates the uptake of peptides into the cytoplasm. (**G**) Very high peptide concentrations may even lead to membrane micellation.

(Figure 5D) and lateral diffusion facilitates the assembly of transient pores [111]. Regardless how they are formed in detail, the barrel stave pores (Figure 5E) comprise five to ten α-helices oriented perpendicular to the membrane surface [27,112]. Typically, the barrel stave mechanism therefore requires helices long enough to span the entire membrane [112]. In the pore, neighboring peptides are directly in contact with each other. While hydrophilic residues are pointing inwards, the hydrophobic outside of the pore is surrounded by the lipid tails [112].

Initially, the barrel stave model was the only model to describe the characteristics of pore formation and was thought to apply to not only alamethicin but also cationic αAMPs such as melittin, cecropins, or magainins [112]. However, with the identification of other pore-forming mechanisms (described below), the applicability of the barrel stave model to cationic αAMPs was increasingly doubted [108,113–116]. For instance, other mechanisms gave better explanation for rapid lipid flip-flopping that was observed with cationic αAMPs [113]. Further, the strong electrostatic repulsion between heavily charged cationic helices would prevent the hydrophobicity-driven peptide–peptide interactions necessary to form the pore [115]. Therefore, the barrel stave mechanism is only applicable to highly hydrophobic peptides with only limited net charge.

Carpet mechanism

Lack of evidence supporting the barrel stave mechanism led to the proposal of a different, much simpler mechanism, in which the peptides accumulate at the outer leaflet of the membrane, oriented in parallel to its surface [116,117]. This model was first proposed in 1988 [117] and subsequently named carpet-like mechanism [115,116,118] or simply carpet mechanism [71,119]. According to the carpet mechanism, monomeric and unstructured αAMPs (Figure 6A) diffuse to the bacterial cytoplasmic membrane, where they accumulate attracted by electrostatic interactions (Figure 6B). While the polar residues remain in the head group region of the membrane, the hydrophobic residues interact with the lipid tails which results in the formation of an α-helical conformation (Figure 6C). Accumulation of αAMPs in the membrane takes place until the membrane is covered by a dense layer of αAMPs [71,117,118]. Calculations based on partition constants and MIC values of several AMPs demonstrate that high membrane coverage is physiologically relevant [120]. It leads to membrane thinning and weakening, as the embedded AMPs push apart the lipid head groups [121,122] (Figure 6C). Then, peptides diffuse through the membrane (Figure 6D) mainly driven by the high transmembrane potential of bacterial cells, effectively perturbing the membrane at the outer and the inner leaflet. With a weakened membrane, flip-flopping of lipids between the inner and outer leaflet of the membrane is facilitated leading to a reorganization of the membrane [71,118,123]. Weakening of the membrane may also result in transient pores or ruptures (Figure 6E) which allow the passage of peptides into and cytosolic compounds out of the

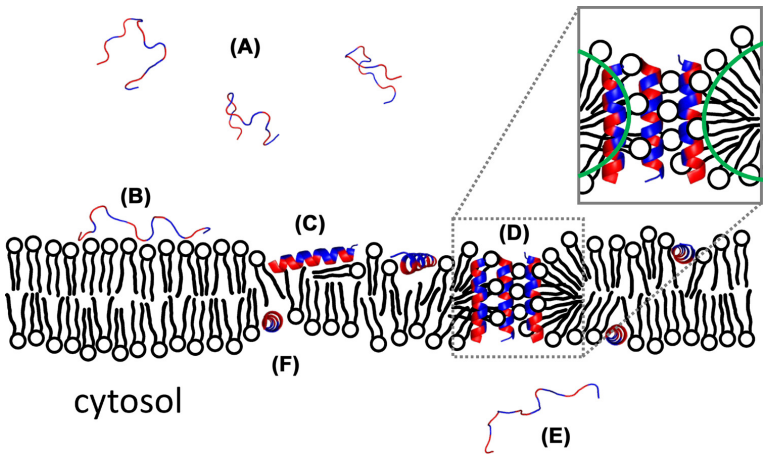

Figure 7. Toroidal pore mechanism

Hydrophilic residues of the peptides are colored in blue, hydrophobic residues in red. The phospholipids of the membrane are represented by black circles (head groups) and two wavy black lines (hydrocarbon tails). The expansion shows a cross-section of the toroidal pore. The green lines indicate the pore lining which connects the outer and the inner leaflet of the membrane. Letters (A–F) describe critical steps in the temporal progression of the pore formation. (**A**) Cationic αAMPs are monomeric and unstructured in solution. (**B**) They are attracted to the membrane surface by charge–charge interactions. (**C**) Insertion into the membrane and formation of α-helical structure is driven by hydrophobic interactions. (**D**) Accumulation leads to the formation of toroidal pores in which the peptides do not directly interact with each other, but are separated from one another by the lipid head groups. (**E**) Pore formation facilitates the diffusion of peptides into the cytoplasm. (**F**) Peptides may also diffuse along the pore lining from the outer to the inner leaflet.

cell (Figure 6F). According to the carpet mechanism, membrane micellation or lysis is often the final result once a critical concentration of peptides is reached [123] (Figure 6G).

Toroidal pore mechanism

In 1996, the toroidal pore mechanism was independently proposed by two research groups for the cationic αAMP magainin 2 [113,114]. This model delivered the explanation for the observation of cationic αAMPs to be oriented perpendicular to the membrane surface, forming large pores despite their electrostatic repulsion [114]. Like the carpet mechanism, the toroidal pore mechanism begins with monomeric αAMPs accumulating at the outer leaflet of the cytoplasmic membrane assuming an α-helical structure oriented parallel to the membrane surface (Figure 7A–C) [114,124]. At high peptide concentrations and subsequent to thinning of the membrane, αAMPs begin to insert vertically into the membrane [114,124]. This results in the formation of toroidal pores (Figure 7D), the walls of which are composed of the peptides oriented perpendicular to the surface and the lipid head groups between the peptide helices [114]. Consequently, the lipids in the toroidal pore connect the inner and outer leaflet forming a single surface (Figure 7, expansion). The supramolecular complex of anionic lipid head groups and cationic peptides is able to shield the electrostatic repulsion between the peptides. As a consequence, there are no or only limited peptide-peptide interactions in the toroidal pore, which is in contrast with the barrel stave model [114]. Connection of the inner and outer membrane leaflet by the pore enables rapid lipid flip-flopping [113,114]. Further, the pore allows the passage of peptides into and cytosolic compounds out of the cell (Figure 7E). Peptides will diffuse to the inner membrane leaflet, effectively weakening the membrane at the outer and the inner leaflet (Figure 7F). The stability and size of the pores are influenced by various factors, such as the properties of the peptide, peptide concentration, the membrane composition, or the transmembrane potential [78,125,126]. In turn, pore stability and size have a direct influence on the pore selectivity and peptide translocation across the membrane [126].

Aggregate mechanism

Initially described as a refinement of the toroidal pore mechanism, the aggregate model was published in 1999 by Robert E. W. Hancock and co-workers to describe the mode of action of short AMPs that are not long enough to span the entire membrane and therefore cannot form an ordered toroidal pore [78]. The aggregate model describes a more disordered state of the lipid-peptide supramolecular complex, which is similar to the toroidal pore with respect to

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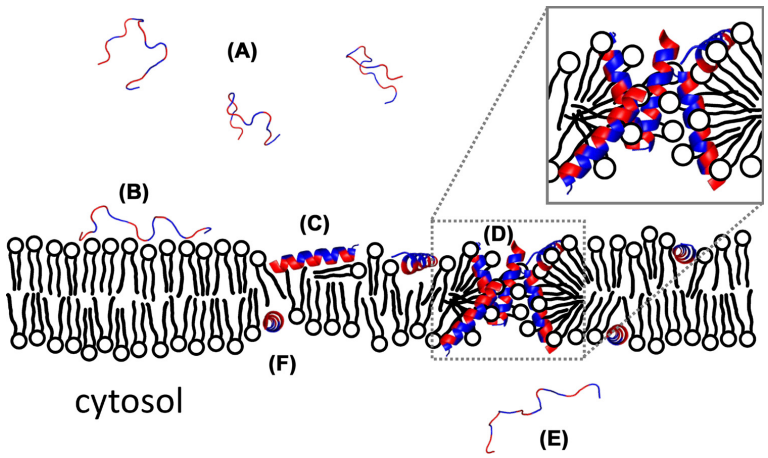

Figure 8. Aggregate mechanism

Hydrophilic residues of the peptides are colored in blue, hydrophobic residues in red. The phospholipids of the membrane are represented by black circles (head groups) and two wavy black lines (hydrocarbon tails). The expansion shows that the peptides in the aggregate are not all vertically aligned with respect to the membrane surface, which is in contrast to the toroidal pore model. Letters (A–F) describe critical steps in the temporal progression of the aggregate formation. (**A**) Cationic αAMPs are monomeric and unstructured in solution. (**B**) They are attracted to the membrane surface by charge-charge interactions. (**C**) Insertion into the membrane and formation of α-helical structure is driven by hydrophobic interactions. (**D**) Accumulation leads to the formation of disordered pores in which the peptides do not directly interact with each other, but are separated from one another by the lipid head groups. The peptides within the pore are not necessarily vertically aligned, but form more or less irregular aggregates with the lipids. (**E**) Aggregate formation facilitates the uptake of peptides into the cytoplasm. (**F**) Peptides may also diffuse along the pore lining from the outer to the inner leaflet.

the arrangement of the lipids, but the peptides are not necessarily vertically aligned [78] (Figure 8D). All other steps (Figure 8A–C,E,F) are identical to the toroidal pore model. The variability in size and stability of those aggregates explain the frequently observed fluctuations of conductance in planar bilayer studies, as well as rapid lipid flip-flop and passage of AMPs across the membrane [78]. A minireview by Robert E. W. Hancock and Daniel S. Chapple revisits the aggregate model rephrasing the aggregate as a 'micelle-like complex' spanning the membrane [127]. The term 'micelle-like complex' emphasizes the disorder of the aggregate and implies that a pore, i.e. an open channel crossing the membrane, is not necessarily present.

Detergent-like mechanism

Over time, the different models were refined and expanded to include new scientific evidence which led to their gradual assimilation and to inclusion of all experimentally observed phenomena in comprehensive models [70,71,128–130]. By proposing micelle-like complexes, the aggregate model uses analogies to detergents [127]. However, detergent-like properties of αAMPs have more often been associated with the carpet model [27,28,71,119,128,129,131], because lysis of the membrane is the final step in this model. In their review from 2006, Burkhard Bechinger and Karl Lohner emphasize the detergent-like bio-physical properties of linear amphipathic cationic AMPs [130]. Their proposed detergent-like model rationalizes the effect of those peptides on a lipid membrane on the basis of their molecular geometries and amphipathicity [130]. For instance, the similar membrane-permeabilizing activities of the αAMP magainin 2 and the surfactants Triton X-100 and octyl glucoside were reported early [132]. According to the detergent-like model, all three molecules have a more or less similar geometry, simplistically described as cone-shaped with a short or shallow hydrophobic part and a large hydrophilic portion [130]. Upon insertion into the membrane, they push apart the hydrophilic head groups of the lipids while being not able to fill the void space they create in the hydrophobic tail region. In consequence, they create a positive curvature strain on the membrane [130,133–135].

The detergent-like model illustrates the complexity of intermolecular and supramolecular interactions in aqueous mixtures of peptides and lipids using phase diagrams [130,135]. An exemplary two-dimensional phase diagram with the variables 'peptide concentration' (ordinate) and 'lipid composition' (abscissa) is shown in Figure 9. Dependent on those two variables, peptide–lipid micellar aggregates, bicelles, lamellar bilayers or hexagonal phases may form

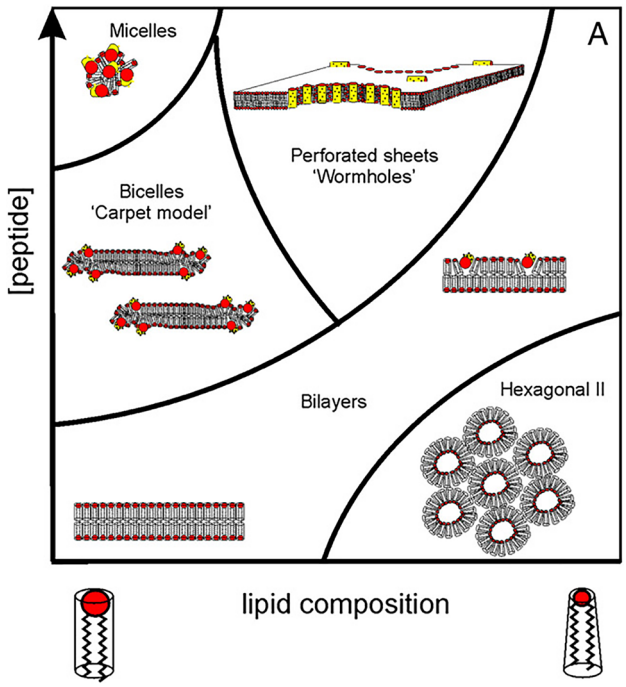

Figure 9. Two-dimensional phase diagram of a mixture of an α AMP with different lipid compositions [135]

Peptides are depicted as yellow cylinders with red basal planes. Cylindrical lipids (i.e. with a large red head group) represent phosphatidylcholines, while inverted truncated cone-shaped lipids (i.e. with a smaller red head group) represent phosphatidylethanolamines. The term 'wormholes' refers to toroidal pores. Reprinted from Current Opinion in Colloid & Interface Science, volume 14, issue 5, Burkhard Bechinger, Rationalizing the membrane interactions of cationic amphipathic antimicrobial peptides by their molecular shape, pages 349-355, Copyright 2009, with permission from Elsevier.

[130]. The toroidal pore, a carpet of peptides covering the membrane or peptide aggregates inside the membrane also each have their own regions in the phase diagram and are considered special cases that occur under distinct conditions [130,135]. Similarly, membrane micellation and fragmentation as described in the carpet model and its inhibition by cholesterol can be explained with detergent-like properties of AMPs [77,135]. Notably, the detergent-like mechanism is also able to rationalize transient membrane disruptions at low peptide-to-lipid ratios, which other models fail explain. When AMPs partition into the outer leaflet of the membrane they perturb the lipid order in a radius of a few nanometers around them. At low peptide-to-lipid concentration, lateral diffusion of the peptides may result in stochastic increases of the local concentration leading to an overlap of regions of reduced membrane stability and formation of transient membrane ruptures [130,136]. Another appealing feature of this model is its tunable complexity. In theory, more variables such as peptide composition, lipid concentration, temperature, pH, or salinity can be added generating a multidimensional phase diagram in which each variable is represented in one dimension.

Multi-target mechanism

A membrane-permeabilizing mode of action can be ascribed to almost any cationic AMP at high concentration [137,138] and extensive formation of pores or ruptures, regardless of the detailed mechanism, eventually leads to the collapse of the proton motive force, loss of cytosolic components and ultimately to growth inhibition or cell death. However, pore formation or—in extreme cases—lysis of the cell is not always the primary mode of action of an αAMP [128,137]. Many αAMPs have a bactericidal or bacteriostatic effect even at concentrations that are not sufficient for the formation of long-lived pores or ruptures. At these concentrations, the damage dealt to the membrane is not profound enough to explain the antimicrobial effect. However, these conditions do suffice to elicit short-lived membrane lesions that enable the peptides to enter the bacterial cytoplasm. Once inside the cell, many αAMPs may disrupt intracellular processes resulting in the killing of the microbes, as the following examples demonstrate.

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The analysis of four different αAMPs regarding their ability to dissipate the membrane potential of *E. coli* showed that membrane disruption does not necessarily coincide with the MIC [78]. Depending on the peptide, membrane dissipation can occur well above or even below the MIC [78]. The difference between MIC and membrane-depolarizing concentration is particularly large with the cationic, α-helical peptide P-Der, a chimera of pleurocidin and its homologue dermaseptin [138]. Applied at concentrations well above its MIC, P-Der permeabilizes the cytoplasmic membrane of *E. coli* immediately and displays a killing efficiency of 99.8% after 10 min [138]. At its MIC, however, P-Der translocates into the cytoplasm of *E. coli* without depolarizing the cytoplasmic membrane, where it is able to inhibit RNA synthesis [138]. Additionally, in model membranes lipid flip-flopping was observed at concentrations that did not cause membrane permeabilization. Both effects, RNA synthesis inhibition and induction of lipid flip-flop, may contribute to its bacteriostatic effect on *E. coli* at P-Der's MIC, but other internal effects may also be involved [138].

The αAMP buforin II is particularly well characterized and highly active against Gram-positive and -negative bacteria as well as fungi [139,140]. Similar to P-Der, it does not lyse or perforate the cell membrane of *E. coli* or *B. subtilis* even at concentrations five times its MIC [140,141]. Supposedly, a kink in its helix [92] induced by a proline residue is responsible for its non-pore-forming translocation across the cytoplasmic membrane [140,142]. In the cytoplasm, it is believed to act by binding to the negatively charged DNA and RNA [140–143].

Another well-studied αAMP with antimicrobial and anti-tumor activity but low hemolytic effect is NK-18 [144,145]. It was shown to act on *E. coli* and *S. aureus* through a membrane-permeabilizing mechanism [145]. In addition, high affinity to plasmid DNA was evidenced *in vitro* [145]. Together, these findings indicate a double-action mechanism targeting the membrane and the DNA.

The αAMP SP1-1 has a unique mode of action as it targets the serine kinase RsbW with nanomolar affinity [146]. Association of SP1-1 with this enzyme compromises RsbW's inhibition of the transcription factor SigB in *S. aureus*, leading to unregulated expression of downstream genes [146]. In the cytoplasm, SP1-1 is also thought to bind bacterial plasmid DNA as *in vitro* DNA-binding assays suggest [146]. Originally designed as an agent for plant protection, the non-hemolytic peptide SP1-1 was tested for pore-forming activity against the bacteria *Pseudomonas syringae pv. syringae* and *Clavibacter michiganensis ssp. michiganensis* resulting in concentration-dependent, but rather low membrane depolarization [147]. Altogether, with some membrane activity, the ability to bind DNA, and high affinity to an important gene expression-regulating enzyme, the peptide SP1-1 makes a compelling case for a multi-target mechanism.

Indications for multi-target mechanisms of prototypical αAMPs were also found in a study analyzing the mode of action of αAMPs designed from a sequence template [148]. The peptides elicit a membrane disruptive mode of action in *Staphylococcus simulans* (*S. simulans*), but hit *S. aureus* differently: Concentrations 10-fold the MIC against *S. aureus* had only little impact on the bacterium's transmembrane potential and did not evoke release of radioactively labeled glutamine from the cytosol. Active glutamine uptake, however, was largely inhibited after treatment with the peptides [148]. Analysis of gene expression patterns before and after exposure to sublethal doses of one αAMP in a different *S. aureus* strain revealed that the peptide produced diverse stresses at the plasma membrane affecting cell wall synthesis, lipid metabolism and aerobic energy generation [148].

These examples indicate that other targets than the cell membrane(s) may be as important for the mode of action of cationic αAMPs as their membrane disruptive function [128,137,148]. At concentrations around the MIC, αAMPs do not necessarily permeabilize the membrane which indicates an intracellular mechanism. Even magainin 2, an αAMP with a high pore-forming activity [113,114] that was thought to act solely through membrane permeabilization [125], is able to translocate into the cytoplasm of *E. coli* at concentrations below its MIC [149]. Presumably, cationic αAMPs reach the cytoplasm where they unspecifically bind to DNA or RNA through electrostatic interactions and thus interfere with macromolecule synthesis in the bacterium. Furthermore, non-membrane permeabilizing interactions of AMPs with the cytoplasmic membrane potentially influence lateral diffusion, disturb the organization of membrane domains or disorganize the leaflet asymmetry. Mounting evidence for multi-target modes of action lead to the conclusion that cationic αAMPs in general follow a mechanism aptly described as 'sand in a gearbox' [148] employing multiple low-affinity interactions with the microbial membrane(s) as well as internal targets [128,137,145,148]. Like a distributed denial of service attack, each individual interaction is harmless to the bacterium, but taken together the simultaneous attacks are overwhelming the bacterium's defense mechanisms. Because many of these interactions may differ among bacterial species, a peptide may confer different mechanisms on different bacteria [148].

Fibrillary structures

The β-amyloid peptide, also referred to as amyloid-β (Aβ), is a peptide that is able to self-associate in a β-sheet conformation to form fibrillary structures found in brain plaques of patients with Alzheimer's disease. Protofibrils of

Aβ, i.e. short fibrillary aggregates with rather low molecular weights, were found to be cytotoxic [150]. Recently, it was shown that these fibrillary aggregates have membrane-permeabilizing capacities [151]. Moreover, antimicrobial properties have been demonstrated for a variety of amyloid peptides [152]. The similarities between amyloid peptides and AMPs become even more obvious, when considering that certain β-sheet antimicrobial peptides are also capable of forming fibrillary structures [150,153–155]. More surprising, however, is the fact that β-sheet structure is not a prerequisite for fibril formation. The following examples show that even αAMPs may spontaneously form amyloid fibrils and raise the question whether the formation of fibrils is a mode of action of αAMPs.

The 17-residue αAMP uperin 3.5 (GVGDLIRKAVSVIKNIV-NH_2) was discovered in skin secretions of the Australian toadlet *Uperoleia mjobergii*. The peptide is prone to forming amyloid fibrils at neutral pH at concentrations of 0.5 μM [156], which is well below its MIC against several Gram-positive bacteria [157]. While pores were formed by uperin 3.5 in lipid bilayers mimicking mammalian cytoplasmic membranes, bacterial membrane mimics were disintegrated by uperin 3.5 [158]. The type of membrane activity of uperin 3.5 was independent from its aggregation state, although monomeric uperin 3.5 was observed to have faster kinetics [158] and lower MICs [157]. Incubation of uperin 3.5 in the presence of the Gram-positive bacterium *M. luteus* or small unilamellar vesicles (SUVs) mimicking a bacterial membrane induced fibril formation [157]. Surprisingly, uperin 3.5 was found to form two kinds of fibrillary structures: cross-α and cross-β fibrils, which were composed of monomers in α-helix or β-sheet conformation, respectively [157]. While the cross-β fibrils formed upon heating of uperin 3.5 and were relatively inactive, the formation of cross-α fibrils was induced not only from monomers, but also from β-fibrils, both in presence of a microbial membrane [157]. The fibril formation was hypothesized to be of biological relevance for the toadlet. Possibly, monomeric uperin 3.5 is secreted constantly and when no bacteria are present, the peptide may adopt its cross-β fibrillary structure to be stored safely. In the presence of bacteria, however, cross-β fibrils may be converted into active cross-α fibrils [157]. It remains unknown whether the cross-α fibrils have direct antimicrobial activity, or whether they just facilitate the release of monomeric uperin 3.5 which then targets the bacterial membrane.

Similar to uperin 3.5, the αAMP human cathelicidin LL-37 was also shown to be able to form fibrillary structures. The presence of 10 μM LL-37 drives the aggregation of liposomes composed of saturated phospholipids into tubular structures with a diameter of approximately 10 μm [159]. Analysis of the liposome-peptide interaction by transmission electron microscopy (TEM) showed fibrillary structures with a diameter of approximately 10 nm, i.e. thousand-fold smaller than the macroscopic tubular structures. Those fibrils formed only in the presence of saturated phospholipids and were assumed to drive the formation of the tubular superstructures from liposomes via an unknown mechanism [159]. No nanoscale fibrils were formed when LL-37 was mixed with unsaturated phospholipids or lipid mixtures containing cholesterol [159].

Like uperin 3.5, the peptide LL-$37_{17\text{-}29}$ (FKRIVQRIKDFLR), a fragment of human LL-37, forms thermostable fibrils in the presence of the *M. luteus*. The fibrils aggregate into ribbons with a few hundred nanometers in width and several micrometers in length [160]. A crystal structure of the fibrils surprisingly indicated that the peptides are incorporated in an α-helical conformation [160]. The surface of the fibrils exhibits regular patterns of hydrophilic and hydrophobic patches which are thought to be responsible for their ability to interact with bacterial membranes [160].

The ability to shift between α-helical and β-sheet structure has not only been observed in uperin 3.5, but also in the αAMP GL13K (GKIIKLKASLKLL-NH_2) [161]. It was shown to form fibrils under basic conditions and during the process, its secondary structure was observed to shift in a pH-dependent manner from unstructured to β-sheet to α-helical [162]. Although the bacterial susceptibility assays were performed in conditions that were too acidic to observe fibril formation, an impact of fibril formation on the MIC has been postulated by the authors [162]. Investigations using nuclear magnetic resonance (NMR) [161] and circular dichroism (CD) [163] spectroscopy revealed formation of β-sheet structures in the presence of biomembrane mimics, but did not inform about the aggregation state. In consequence, the importance of fibril formation for biological activity of GL13K remains inconclusive.

These examples show that fibril formation by AMPs is a topic of active research. Several observations argue against fibril formation being a mode of action. In uperin 3.5 and its derivatives, for example, the propensity to form amyloid fibrils was shown to be inversely correlated with their membrane disrupting activity [158]. Moreover, monomeric uperin 3.5 was observed to have faster membrane-permeabilizing kinetics [158] and lower MICs [157] than in the fibrillary state. Most importantly, formation of fibrils often requires hours, while other antimicrobial peptides have been reported to kill bacteria [148,164–166] or to dissipate their membrane potential [78] within minutes. Therefore, the formation of cross-α fibrils may plausibly also take place after the microbe was killed by the non-fibrillary uperin 3.5.

Other observations indicate that amyloid fibrils have direct antimicrobial activity. For instance, uperin 3.5 and LL-37 form fibrils which are nucleating at the microbial membrane surface. The direct interaction of the membrane and the fibril may plausibly negatively affect the bacterial membrane integrity. Most amyloid peptides are not cytotoxic

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in their monomeric state or when self-assembled into fibrils, but only in the protofibrillary state [150]. Potentially, the formation of protofibrils nucleating at the bacterial membrane surface may have a bactericidal effect. Unfortunately, the causality between antimicrobial action and (proto)fibril formation is difficult to prove and future studies are required to determine whether formation of fibrils or protofibrils truly is a mode of action of AMPs or just a peculiar phenomenon.

Anti-biofilm activity

In need of compounds for the treatment of biofilm infections, many antimicrobial peptides have been investigated regarding their ability to prevent biofilm growth or eradicate established biofilms [167]. Basically any compound with a bactericidal effect should be able to prevent the initial colonization of a surface, because the planktonic bacteria that first attach to a surface do not benefit from protection by an EPS matrix. Indeed, AMPs proven to be active against planktonic bacteria have been shown to prevent biofilm formation on medical devices like catheters in animal models [168,169]. The eradication of mature biofilms, however, is of greater clinical relevance and more complex. In this regard, the high net positive charge of cationic AMPs may be a potential drawback. The cationic AMPs may be sequestered by the anionic biofilm matrix requiring concentrations above the binding capacity of the matrix for the peptides to reach the bacteria. On the other hand, the ability of AMPs to damage bacterial membranes is a mode of action not only affecting metabolically active cells but also persisters. For instance, the αAMP DFT561d (GLKLLLSLGLKLL-NH_2, all-D-residue-peptide) proved efficiency in eradicating 24 h old biofilms of methicillin-resistant *S. aureus* (MRSA) *in vitro* [170]. Importantly, it even achieved killing of nafcillin-induced persisters, which is interpreted as an indication for activity against biofilm-induced persisters [170].

When applied at 16 μM (twice the MIC against planktonic *P. aeruginosa*), the peptide PaDBS1R7 (PMARNKPKILKRILAKIFK-NH_2) was able to eradicate 2 d old *P. aeruginosa* biofilms in flow chambers [171]. Moreover, this peptide was applied in a skin abscess mouse model at 64 μM, where it was able to reduce the bacterial burden by a factor of 100 to >1000 four days after treatment with a single dose of peptide [171]. In addition to reducing the bacterial load, this peptide showed immunomodulatory activity facilitating a quick recovery from the infection [171].

Surprisingly, biofilm dispersal may be achieved by αAMPs even below their MIC. The most prominent example is the HDP LL-37, which achieves a reduction of an established *P. aeruginosa* biofilm by 60% at concentrations below 1 μM [172]. The presence of LL-37 induces the expression of genes responsible for twitching motility and suppresses the expression of genes involved in quorum sensing [172]. Both effects hamper the formation of biofilms by *P. aeruginosa* and result in biofilm dispersal [172]. Similarly, the peptide IDR-1018 (VRLIVAVRIWRR-NH_2) [173] triggers biofilm dispersal of a variety of pathogens including *P. aeruginosa*, *Acinetobacter baumannii* (*A. baumannii*), *Klebsiella pneumoniae* (*K. pneumoniae*), and MRSA [174]. In the case of *P. aeruginosa*, IDR-1018 concentrations of 0.5 μM induced dispersal of a 2 d old biofilm *in vitro*, while a concentration of 6.5 μM had a bactericidal effect [174]. IDR-1018 was found to bind the second messengers guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and guanosine 5′-triphosphate 3′-diphosphate (pppGpp), which are known to regulate the expression of biofilm-related genes (among others) in bacteria [174]. Supposedly, IDR-1018 is able to traverse the bacterial membranes and enter the cytoplasm, where binding of those second messengers leads to their degradation. Depletion of cellular (p)ppGpp levels then triggers a signal cascade to initiate biofilm dispersal [174]. When IDR-1018 was applied together with conventional antibiotics like ciprofloxacin, synergistic effects were observed facilitating the eradication of mature biofilms [175].

Peptide design

The goal of many AMP design studies is to provide highly active, non-toxic AMPs that have a high bioavailability, are cheap to produce, and able to kill antibiotic-resistant pathogens *in vivo*. Understanding which parameters affect an AMP's observable macroscopic properties including activity against different pathogens, hemolysis, toxicity against mammalian cells, or bioavailability is crucial for the design of active, peptide-based antibiotics. In general, the observable macroscopic effects of AMPs result from intermolecular interactions dictated by molecular properties which in turn are determined by the sequence of amino acids. The intermolecular interactions imply phenomena as peptide–membrane interactions, peptide aggregation, enzymatic degradation, or peptide–DNA interactions. Molecular properties include among others hydrophobicity, amphipathicity, net charge, helical propensity, peptide length, and structural characteristics such as kinks or unfolded regions. While the molecular properties of αAMPs are relatively easy to deduce from a given short primary structure, the countless intermolecular interactions *in vivo* add such complexity to the system that the resulting macroscopic properties are much more difficult to understand in

Table 1 The sequence template by Alessandro Tossi and colleagues [28,105]

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
G	**Θ**	**Θ**	**±** ρ	**+** ρ	**Θ**	**X G**	**±** ρ	**X**	**Θ**	**±** ρ	**+** ρ	**Θ**	**Θ G**	**+** ρ	**+**	**Θ**	**X**	**±** ρ	**G**

This sequence template represents the average N-terminal stretch of more than 150 natural αAMPs of the AMSDb. Each position is marked by one or two symbols indicating the most common type of amino acid in the indicated position: Glycine (**G**), hydrophobic (**Θ**), positively charged (**+**), either positively or negatively charged (**±**), polar (ρ), no preference for any specific residue (**X**).

detail. A review article summarizing the most important computational and non-computational approaches to AMP design was recently provided by Marcelo D. T. Torres, César de la Fuente-Núñez and colleagues [176]. This chapter will reflect on the historic development of αAMP design.

Rational and heuristic design of αAMPs

Soon after the discoveries of HDPs in a variety of species, such as cecropins [177] in hemolymph of cecropia moth (*Hyalophora cecropia*) pupae or magainins [166] in the skin of African clawed frogs (*Xenopus laevis*), synthetic analogs were produced and compared to variants with point mutations or deletions in order to figure out what sequential characteristics determine antimicrobial activity [178–180]. From first structure–activity relationship (SAR) studies like these in the 1980s it became apparent that cationicity, helicity, and amphipathicity are important determinants for the activity of αAMPs. With this knowledge, αAMPs were designed *de novo* from regular repeats of cationic and hydrophobic residues [181,182]. Other early αAMP design studies combined different parts of αHDPs creating chimeric peptides with improved properties, concluding that kinks or hinges between two helical stretches may be an important structural feature in cecropins and cecropin-derived peptides [104,183]. In the process of developing broadly potent αAMPs, Magainin Pharmaceuticals, Inc. produced over 200 substitution and deletion variants of magainin and PGLa. Their data set confirmed the importance of basicity and hydrophobicity for activity against a broad spectrum of pathogens, but indicated an increased hemolytic activity when the hydrophobicity was too pronounced [184].

In the 1990s, new ideas inspired the design of αAMPs. For instance, short amphipathic and cationic helical motifs were derived from larger proteins [185,186]. The implementation of synthetic combinatorial libraries (SCLs) was another significant innovation in αAMP design at that time [187,188]. SCL approaches can yield active and selective αAMPs [188] but synthesis and screening of SCLs is a complex endeavor and extracting information from SCLs requires elaborate deconvolution techniques [187,188]. In consequence, this approach is not easily implemented in laboratories and has not been applied in many studies.

In the late 1990s and in the 2000s, more systematic attempts were made to quantitate the influence of molecular parameters on the antimicrobial activity and cell selectivity of αAMPs, such as hydrophobicity [189–191], hydrophobic moment [189,190,192], (inducible) helicity [189,190,193,194], charge [189], and angle subtended by hydrophobic or charged residues [126,190,195]. For instance, a non-continuous hydrophobic face interrupted by a hydrophilic residue was shown to reduce the hemolytic effect of αAMPs [55,196]. Similar approaches to identify the influence of molecular parameters were conducted using data base-supported design of αAMPs [28,105,148,197,198], facilitated by the many sequences of αAMPs and αHDPs that had been identified and archived by that time. Notably, Alessandro Tossi and co-workers evaluated a data set of more than 150 natural αAMPs within the Antimicrobial Sequences Database (AMSDb; now offline) and gained empiric information on the typical ranges of the parameters net charge, content of hydrophobic residues, and amphipathicity [28,105]. They created a sequence template based on the first 20 N-terminal residues of each database entry (Table 1) [28,105]. The N-terminal domain was chosen, as it 'is generally essential and often sufficient for antimicrobial activity' [28].

With this template a series of 20 systematically altered, short peptides was synthesized and characterized in order to determine the influence of the individual molecular parameters on the macroscopic properties [28,105]. Unfortunately, it is hardly possible to modify one molecular property without affecting others when using only the standard 20 amino acids. For example, an Ile→Lys mutation not only affects the net charge, but also the hydrophobicity, amphipathicity and helical propensity. Therefore, non-standard residues were incorporated to uncouple the different molecular properties from each other and to identify their individual importance for antimicrobial activity [28,105].

Computational design of αAMPs

Since the turn of the millennium, increasing computing power combined with growing data bases collecting structural and activity data on AMPs and HDPs facilitated the development of computational approaches to αAMP design

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[176,199]. The discovery of AMPs in previously uncharted sequence space, i.e. design of AMPs with no or only limited sequence identity to known AMPs, is the goal of many computational design studies. Those *de novo* design strategies, also referred to as quantitative SAR (QSAR) studies, are commonly based on a set of AMPs that is statistically evaluated based on so-called descriptors, i.e. measurable or computable properties that are responsible for or at least co-occur with antimicrobial activity. Found correlations are then used to predict the activity of novel peptides. The accuracy of the prediction is highly dependent of the size (and accuracy) of the original dataset, the chosen descriptors, and the statistical method. To obtain a very large training dataset, two landmark articles [200,201] from 2009 reported the use of high-throughput methods [202] to synthesize >1400 AMPs and to analyze them regarding their antimicrobial activity. The resulting dataset was used as the input for artificial neural networks (ANNs) to predict the antimicrobial activity of 100,000 hypothetical antimicrobial peptides that are each nine residues in length [200,201]. Two lead candidates, the αAMPs HHC-10 (KRWWKWIRW) and HHC-36 (KRWWKWWRR) [200], were synthesized and showed high *in vitro* activity against a variety of pathogenic bacteria. They were even successfully applied in a murine *S. aureus* infection model [200].

An important computational method is pattern recognition, which scans datasets of AMPs for common patterns in their sequences. For instance, the α-core formula, a sequence template similar to the one developed by Alessandro Tossi and co-workers, was found by pattern recognition. The α-core formula was then used to screen protein sequences archived in data bases of the public domain in order to identify protein fragments with predicted antimicrobial activity [203]. Pattern recognition was also involved in an early method of computational AMP design which has been introduced as a 'linguistic model' because the detected patterns of residues in a set of natural AMPs resemble the grammatical rules of a language [204]. A similar pattern recognition method was also used in a design algorithm called Joker. The Joker algorithm was used to convert inactive peptides into antimicrobial peptides by introducing mutations according to patterns identified in a dataset of antimicrobial peptides [205]. Similarly, a pattern recognition algorithm has been used for the *de novo* design of αAMPs [206]. A different study sought to design highly selective αAMPs based on a dataset comprising 73 frog-derived AMPs with published antimicrobial and hemolytic activities. In this study, pattern recognition was combined with other descriptors including the so-called sequence moment [56]. This descriptor is a vector representing the asymmetry of a peptide's hydrophobicity along its sequence. The sequence moment was shown to correlate with a peptide's therapeutic index, i.e. its selectivity toward bacterial cells over erythrocytes. The algorithm produced the αAMP adepantin 1 that was shown to exhibit high activity against Gram-negative bacteria and a low hemolytic behavior [56]. Remarkably, although using only amphibian AMPs as input, adepantin 1 does not resemble any natural AMP [56].

Genetic algorithms, also referred to as evolutionary algorithms, are peptide design methods inspired by Darwin's theory of evolution. Computer-aided genetic algorithms have been applied already in the 1990s [207], but more recent approaches are based on much larger datasets and more complex fitness functions [208,209]. In general, genetic algorithms are based on a peptide which is subject to mutation resulting in a set of mutated peptides. The peptides in this new set are evaluated with regard to a certain property, e.g. antimicrobial activity. The best peptides are selected for further mutation and peptides that do not fit the selection criteria are discarded. The mutation/selection cycle is repeated until a peptide has evolved that fits the required properties, e.g. high antimicrobial activity. The approach may be pursued entirely without computational aid, but this would imply the synthesis and testing of hundreds or thousands of peptides. To reduce time and expense, instead of introducing random mutations, mutations may be directed by a machine learning algorithm which increases the probability of obtaining peptides with the desired property. This principle was tested when the AMP temporin-Ali (FFPIVGKLLSGLL-NH_2) [210] was subject to two almost identical genetic algorithms. Their only difference was that in only one of them the mutations were directed by a machine learning algorithm, that would use the previously synthesized and characterized generations of peptides as training input [208]. When the mutations were selected by the machine learning algorithm, the average activity of each generation improved and finally converged at the third generation [208]. In contrast, when mutations were implemented randomly, each generation contained only a few highly active peptides but also many peptides with low or no activity [208]. It should be noted, however, that the most active peptides from either strategy were similarly active with MICs against *E. coli* ranging between 0.5 and 2 μM [208]. This study showed, that machine learning may be applied together with synthesis and testing to reduce the amount of inactive sequences resulting from evolutionary peptide design. However, computational methods may also be used for the *in silico* evaluation of randomized mutants to entirely replace the experimental synthesis and testing during each cycle, as was shown by a study starting from four fragments of the plant AMP Pg-AMP1 [209]. The mutation/selection cycles were performed entirely *in silico* using a so-called fitness function based on the hydrophobic moment and helical propensity [209]. Starting from the same sequence, 100 independent simulations with 50 mutation/selection cycles each were performed resulting in a total of 100 final sequences. Peptides reflecting the 15 highest ranked sequences were synthesized and assayed *in vitro* [209].

The best-performing candidate was guavanin 2, a 20-residue αAMP that primarily targets Gram-negative bacteria *in vitro* and in mouse models and acts through a membrane-permeabilizing mechanism [209].

With increasing datasets, it became apparent that there is no universal approach to design the most potent, least toxic antimicrobial peptide with broad-spectrum activity. Realizing the necessity for selectively active antibiotics, many researchers turned to developing αAMPs targeting only a specific group of pathogens. For instance, rational design by targeted mutation of a broadly potent, membrane-permeabilizing αAMP led to the discovery of a peptide targeting primarily Gram-negative bacteria [196]. In a different case, the research group of Guangshun Wang, the developer and curator of the antimicrobial peptides database (APD) [211,212], developed a computational database filtering technology to design a highly potent αAMP against MRSA [213]. In this method, descriptor-based selection algorithms take into account the peptide length, charge, hydrophobicity, common residues, and secondary structure of active AMPs to identify important residues to create an AMP with selectivity for Gram-positive bacteria. Subsequently, the identified residues were arranged into a peptide sequence according to a pattern analysis similar to a linguistic approach [213]. Local sequence shuffling slightly increased the antibacterial activity, but more importantly drastically reduced the hemolytic effect, because of a non-continuous hydrophobic face of its α-helix [170]. The resulting αAMPs DFT503 (GLSLLLSLGLKLL-NH_2) and DFT561 (GLKLLLSLGLKLL-NH_2) proved efficient in reducing the bacterial burden in neutropenic, i.e. immunosuppressed, mice infected with MRSA [170]. It was further shown that DFT503 would also enhance the immune reaction of non-neutropenic mice [170]. Remarkably, DFT503 and DFT561 have a net charge of only +2 and +3, respectively, and increasing the net charge did not only increase the hemolytic effect but also resulted in a complete loss of efficiency *in vivo* [170].

α-helical AMPs: From bench to bedside?

Resistances of bacteria against AMPs occur less often than against conventional antibiotics because the mode of action of AMPs is not to block a certain receptor that may be altered as a consequence random mutations, but to employ a multi-target mechanism [214,215]. In consequence, those peptides have drawn the interest of academic research laboratories and of pharmaceutical companies [18,22,25,96,97]. As early as 1987, Michael Zasloff, the discoverer of magainins, was one of the first researchers to recognize the 'therapeutic potential [of antimicrobial peptides] in the treatment of bacterial, fungal, and protozoan infections in man' [166]. Indeed, there are many advantages of αAMPs that add to their benefit of eliciting only very few resistances in bacteria. Due to their small size and no requirement for post-synthetic modifications, αAMPs are easy to synthesize and characterize. They may be active against a wide spectrum of pathogens or designed to affect only specific targets. Finally, their peptidic nature renders them non-persistent if released into the environment. Opposing those advantages are several disadvantages that may include systemic or local toxicity, dependence of the activity on salinity or pH value, low bioavailability due to proteolysis or due to adsorption to components of body fluids and cells, development of sensitivities or allergies, as well as high production costs [18]. The transfer from the laboratory (bench) into the clinic (bedside) is only possible, if those disadvantages are addressed and resolved.

Since the early 2000s, enzymatic degradation of αAMPs has been recognized to potentially limit the bioavailability *in vivo* [97,216–219] and strategies to avoid proteolysis of αAMPs are increasingly implemented in the design processes. Such strategies are based on amidation and acetylation of the C- and N-terminus [96,97,220], the incorporation of D-amino acids [96,169,220,221] or other non-standard amino acids [97,169,221,222], the synthesis of peptides with sequences designed to avoid cleavage sites [220,223,224], as well as the stabilization of secondary structure through side chain cross-linking [225–227] (also referred to as peptide stapling).

Protection of the N-terminus by acetylation and of the C-terminus by amidation is inexpensive when peptides are produced by solid phase peptide synthesis (SPPS). These measures provide protection against degradation by exopeptidases, but are inefficient with regard to endopeptidases. In contrast, all-D-αAMPs are not susceptible to enzymatic proteolysis at all [98,99]. Unfortunately, all-D-AMPs are very expensive to produce and their production cost will preclude their commercialization as therapeutics. However, αAMPs do not need to be composed entirely of D-amino acids to be resistant to proteases. Directed incorporation of only a few D- or other non-genetically encoded amino acids was also shown to provide resistance to enzymatic proteolysis [169,221,222,226], but these non-standard residues will also significantly increase the production costs compared with all-L-AMPs. An elegant and inexpensive way to increase the stability of αAMPs with regard to enzymatic proteolysis was achieved by arranging the residues in such a way that trypsin-, chymotrypsin, and aureolysin-specific cleavage sites were avoided [224]. The resulting peptide GNU7 (RLLRPLLQLLKQKLR) was highly active, short, composed of only gene-encoded L-amino acids, and nonetheless highly resistant to a selection of proteases [224]. A different way to increase the resistance of αAMPs against enzymatic proteolysis is peptide stapling. In this approach, the α-helical structure of peptides is stabilized by

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covalently cross-linking the side chains of two residues with *i*, *i*+4, *i*, *i*+7 or *i*, *i*+11 spacing [225]. As proteases require their peptide substrates to be in an extended conformation for efficient cleavage [228], a stable α-helical structure may increase the resistance to enzymatic proteolysis. Indeed, stapled αAMPs have been shown to be highly resistant to proteolytic degradation [226,227,229], which provided the basis for efficacy in animal infection models [226,229]. A bicyclic stapling approach has been reported, tethering three lysine residues positioned at *i*, (i + 4) and (i + 8) [230]. Even though the bicyclic stapled peptide showed a decreased helical propensity compared with its linear parent peptide, the trifunctional staple increased the resistance to enzymatic proteolysis rendering the peptide effective in a murine MRSA infection model [230]. Unfortunately, however, peptide stapling is not accessible through biosynthetic methods.

Recently, miniprotein grafting was used to stabilize the α-helical conformation of the LL-37-derived αAMP KR-12 (KRIVQRIKDFLR-NH_2) in solution [231]. KR-12 was fused to the miniprotein Trp-cage, a 20-residue miniprotein [232,233] with a stable tertiary structure including a short N-terminal α-helix. The Trp-cage domain was originally discovered in the peptide exendin-4 which was found in the oral secretions of the lizard *Heloderma suspectum* [234]. Exendin-4 shows several antidiabetic effects of the human hormone glucagon-like peptide 1 (GLP-1), but has a significantly higher bioavailability [235] and a 30-fold longer half-life in human blood plasma [236]. These effects are the results of a higher resistance against enzymatic proteolysis derived in parts from exendin-4's stable α-helical structure induced by the Trp-cage domain [236]. When KR-12 was fused to a Trp-cage domain, the stability of the Trp-cage carried forward to the KR-12 derived segment of the chimeric miniprotein termed AMTC31-6. Fusion to the Trp-cage had only minor impact on the high antimicrobial activity of KR-12 at low salt concentrations. More importantly, however, AMTC31-6 showed moderate (10 μM) activity against *P. aeruginosa* and high activity against *E. coli* (0.04 μM) at pH = 5.5 at 150 mM NaCl (conditions that rendered KR-12 inactive) whilst having only low activity against human erythrocytes. Additionally, the stabilized α-helical structure in the KR-12-derived segment reduced the susceptibility of AMTC31-6 to degradation by trypsin by a factor of 2.5 at 37 °C compared with KR-12 [231].

Next to enzymatic degradation, toxicity is a potential weakness of αAMPs that needs to be addressed. Because a pore-forming or membrane-disrupting mode of action is assumed for most αAMPs, hemolysis is commonly used as an indicator for toxicity [52]. However, hemolysis assays can at best report on the toxicity with regard to cell membrane permeabilization, but provide no information on the toxicity towards internal targets or immunomodulating effects. In addition, *in vitro* tests are not necessarily well-suited to predict the activity *in vivo*. For instance, the *in vivo* activity may be affected by a low bioavailability as a result of metabolization, degradation, or adsorption to tissues or serum components. Recognizing those point of concerns, more recent studies have evaluated the efficiency of αAMPs in animal models [169–171,200,209,221,226,229,230,237].

Production costs have mainly been addressed by designing short antimicrobial peptides [90,238–240] - especially when techniques such as SCLs [187] or high-throughput synthesis and screening [200–202] are used. Longer peptides are more expensive to synthesize and recombinant production methods [241–244] may become a cost-efficient alternative with increasing length. Of course, measures have to be taken to prevent the antimicrobial peptide from killing the expression organism. Typically, AMPs are therefore produced as fusions with a protein to reduce the antimicrobial activity, increase the solubility, amplify the expression and facilitate the purification [242,244]. Cost-effective industrial scale production of AMPs by means of gene expression is currently restricted to the standard 20 gene-encoded amino acids [243,244]. Several strategies to prevent proteolytic degradation rely on D-amino acids or other non-standard residues and are therefore not compatible with recombinant AMP production. In addition, production costs for peptides or proteins produced by recombinant gene expression are still very high compared with standard antibiotics. Even if the costs for recombinant production of αAMPs could be brought down to the production costs of human insulin (approximately 25,000 US$/kg) [245], they would still by far exceed those of penicillin (20 US$/kg) [246]. In conclusion, peptide antibiotics cannot compete with conventional antibiotics in terms of production costs, so far. Therefore, AMPs will most likely not be such mass products in the next 50 years, but will only be used in cases where cheaper antibiotics fail. In this niche, however, high production costs may be justified and accepted by the public. As a positive side effect of the high costs and the limited cases where the use of AMPs may be indicated, however, their overuse in human medicine will be limited and their extensive use in veterinary medicine will likely be completely avoided for economic reasons. Thus, mistakes that have been made in the past with conventional antibiotics may be prevented with AMPs.

Although most research regarding AMPs and HDPs is academic and/or pre-clinical, some of those compounds have already entered clinical stages [25]. Of course, many compounds have failed to show superior efficiency compared with conventional antibiotics in clinical studies, but many more are currently being developed or are already in clinical trials [25]. The majority of peptides are administered topically, while oral or intravenous administration is rare [25].

Presumably, the relatively high concentrations required for antibiotic activity necessitate the administration of the active compound directly at the site of infection, e.g. in skin or wound infections by topical administration or in lung infections when administered by inhalation.

One αAMP that is quite advanced in clinical trials is OP-145 (Ac-IGKEFKRIVERIKRFLRELVRPLR-NH_2; also referred to as AMP60.4Ac or P60.4Ac) [25]. This LL-37-derived peptide was the active pharmaceutical ingredient (API) of ototopical drops to treat chronic suppurative otitis media (CSOM) in a double-blinded phase IIa clinical trial [247]. The trial was performed with patients suffering from CSOM resistant to standard treatments. Both, antibiotic-resistant strains as well as formation biofilms may contribute to the lack of efficacy of conventional antibiotics against CSOM [247]. The peptide was shown to be safe for use in humans when administered twice daily for 2 weeks and treatment achieved a success rate of 47% compared to 6% in the placebo group [247]. Surprisingly, no major differences in the microbial flora at the site of infection was observed before and after treatment. Potentially, not the antibacterial properties were responsible for improvement in patients, but the peptide's neutralizing effect on the cytotoxic and pro-inflammatory bacterial products such as LPS and lipoteichoic acids [247]. Unfortunately, the precise mode of action cannot be determined from this study. Regardless, the study shows that AMPs may achieve successful treatment of chronic infections where conventional antibiotic therapies fail.

To conclude, many disadvantages of antimicrobial peptides hamper their development into therapeutics. Nonetheless, the hopes of finally obtaining an active antimicrobial drug that produces only few resistances in pathogens and beneficially modulates the immune response of the host continues to motivate researchers to find ways to circumvent those disadvantages and develop efficient peptide-based antibiotics.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Nils Preußke: Conceptualization, Funding acquisition, Visualization, Writing—original draft, Writing—review & editing. **Frank D. Sönnichsen:** Supervision, Writing—review & editing. **Matthias Leippe:** Supervision, Writing—review & editing.

Abbreviations

αAMP(s), α-helical antimicrobial peptide(s); *A. baumannii*, *Acinetobacter baumannii*; ADP, adenosine diphosphate; AMP(s), antimicrobial peptide(s); AMSDb, antimicrobial sequences database; ANN(s), artificial neural network(s); APD, antimicrobial peptides database; API, active pharmaceutical ingredient; ATP, adenosine triphosphate; Aβ, amyloid-β; *B. subtilis*, *Bacillus subtilis*; CD, circular dichroism; CSOM, chronic suppurative otitis media; DNA, deoxyribonucleic acid; DPC, dodecylphosphocholine; *E. coli*, *Escherichia coli*; EHEC, enterohemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; EPS, extracellular polymeric substances; EU, European Union; GLP-1, glucagon-like peptide 1; HDP(s), host defense peptide(s); IDR(s), innate defense regulator(s); *K. pneumoniae*, *Klebsiella pneumoniae*; LPS, lipopolysaccharide(s); *M. luteus*, *Micrococcus luteus*; MHC, minimal hemolytic concentration; MIC, minimal inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramic acid; NMR, nuclear magnetic resonance; *P. aeruginosa*, *Pseudomonas aeruginosa*; ppGpp, guanosine 5′-diphosphate 3′-diphosphate; pppGpp, guanosine 5′-triphosphate 3′-diphosphate; QSAR, quantiative structure-activity relationship; RBC(s), red blood cell(s); RNA, ribonucleic acid; S-layer, surface layer; *S. aureus*, *Staphylococcus aureus*; *S. simulans*, *Staphylococcus simulans*; SAR, structure-activity relationship; SCL(s), synthetic combinatorial librar(y/ies); SDS, sodium dodecyl sulfate; SPPS, solid phase peptide synthesis; SUV(s), small unilamellar vesicle(s); TEM, transmission electron microscopy; TI, therapeutic index, $TI = MHC \cdot MIC^{-1}$.

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