

## Review

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# Defense peptides: recent developments

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**Abstract:** Defense peptides are small amphipathic molecules that exhibit antimicrobial, antitumor, antiviral, and immunomodulatory properties. This review summarizes current knowledge on the mechanisms of antimicrobial activity of cationic and anionic defense peptides, indicating peptide-based as well as microbial cell-based factors affecting this activity. The peptide-based factors include charge, hydrophobicity, and amphipathicity, whereas the pathogen-based factors are membrane lipid composition, presence of sterols, membrane fluidity, cell wall components, and secreted factors such as extracellular proteinases. Since defense peptides have been considered very promising molecules that could replace conventional antibiotics in the era of drug-resistant pathogens, the issue of microbial resistance to antimicrobial peptides (AMPs) is addressed. Furthermore, selected approaches employed for optimization and *de novo* design of effective AMPs based on the properties recognized as important for the function of natural defense peptides are presented.

**Keywords:** amphipathicity; anionic defense peptides; antimicrobial peptides; bacterial resistance.

## Introduction

Defense peptides are usually defined as small, mainly cationic, amphipathic molecules involved in eradication of pathogens invading a multicellular host. Given the well-documented antimicrobial properties, including antibacterial, antifungal, and antiprotozoan activity, the term ‘antimicrobial peptides’ (AMPs) is used interchangeably.

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However, they can also be active against cancer cells and enveloped viruses, and many of them are important modulators of immune response, e.g. as anti-inflammatory factors (1, 2). A multitude of defense peptides have been found in body fluids, epithelial cells, and phagocytes of multicellular animals, including humans. Various AMPs have been also described in plants, fungi, and unicellular organisms, thus covering all three domains of life, i.e. Bacteria, Archaea, and Eukaryota (3–5). While many defense peptides are ribosomally synthesized molecules encoded by specific genes, the others result from controlled proteolytic degradation of larger proteins very often playing distinct functions in an organism, e.g. buforins from histone 2A (6). In addition, small proteins, e.g. RNase A family members, also exhibit antimicrobial activity, and their involvement in anti-pathogen defense has been well documented (7). Usually, a set of defense peptides differing in biochemical properties and mechanism of antimicrobial action is synthesized by a given organism. Such a strategy creates a great diversity of defense peptides whose synergistic action increases considerably the antimicrobial potential of a given species and simultaneously saves energy resources (8–13).

The AMP database CAMP – Collection of Anti-Microbial Peptides – comprises more than 5000 sequences of natural origin as well as designed synthetic peptides (14) ([www.camp.bicnirrh.res.in](http://www.camp.bicnirrh.res.in)). To date, from more than 2500 natural defense peptides deposited in another database, i.e. the Antimicrobial Peptide Database, almost 2300 are of eukaryotic origin, of which 982 have been reported in amphibians, 248 in insects, and 110 in humans (3, 15) (<http://aps.unmc.edu/AP>). Recently, in their excellent review, Xu and Lai (16) have described an impressive number of 1900 gene-encoded AMPs from skin secretions of 178 amphibian species. Many novel defense peptides have been also identified in various marine organisms (17, 18) and insects (19, 20).

Taking into consideration the amino acid sequence and spatial conformation, the defense peptides can be classified into three groups: (i) linear peptides without cysteine residues adopting  $\alpha$ -helical structures, e.g. insect cecropins and frog magainins; (ii) peptides with a structure stabilized by disulfide bridges, e.g. defensins;

and (iii) peptides with a substantial content of one of the amino acids such as proline (Pro; P), glycine (Gly; G), histidine (His; H), and tryptophan (Trp; W). The cationic properties of AMPs in physiological conditions are provided by high contents of arginine (Arg; R) and lysine (Lys; K) residues. However, among defense peptides there are neutral molecules, e.g. *Galleria mellonella* defensins and a cecropin D-like peptide, or even highly anionic ones, e.g. *G. mellonella* anionic peptide 1 and 2, some insect and amphibian defensins, amphibian maximin 5, and human dermcidin (21–25).

## Mechanisms of antimicrobial action of defense peptides

The basic function of defense peptides as effector molecules of animal and plant innate immune response is to prevent infection by direct killing of pathogens. It is generally accepted that the initial step, i.e. binding of cationic AMP to a microbial cell surface, is promoted by electrostatic interactions between the positively charged peptide and the negatively charged microbial cell surface followed by strong hydrophobic interactions between the peptide amphipathic domains and the membrane phospholipids (PLs). Such binding leads to alterations in transmembrane electrochemical gradients, membrane permeabilization, formation of channels, and even fragmentation of the membrane. Several models describe the interaction of AMPs with bacterial cell membranes, e.g. the (i) carpet model, (ii) barrel-stave model, (iii) toroidal pore model, and (iv) aggregate model (Figure 1A) (26). To facilitate lipid membrane penetration, peptide molecules can interact with each other just before the interaction with PL molecules. In their recent study, Wang et al. (27) demonstrated cooperative behavior of frog aurein molecules during the initial step of binding with palmitoylphosphatidylcholine and palmitoylphosphatidylethanolamine lipid bilayers. The peptides formed intermolecular hydrogen bonds, which resulted in formation of hydrophobic aggregates, thereby minimizing the interactions between water and hydrophobic side chains. Upon reaching the water/lipid interface, the aggregates collectively penetrated into the membrane.

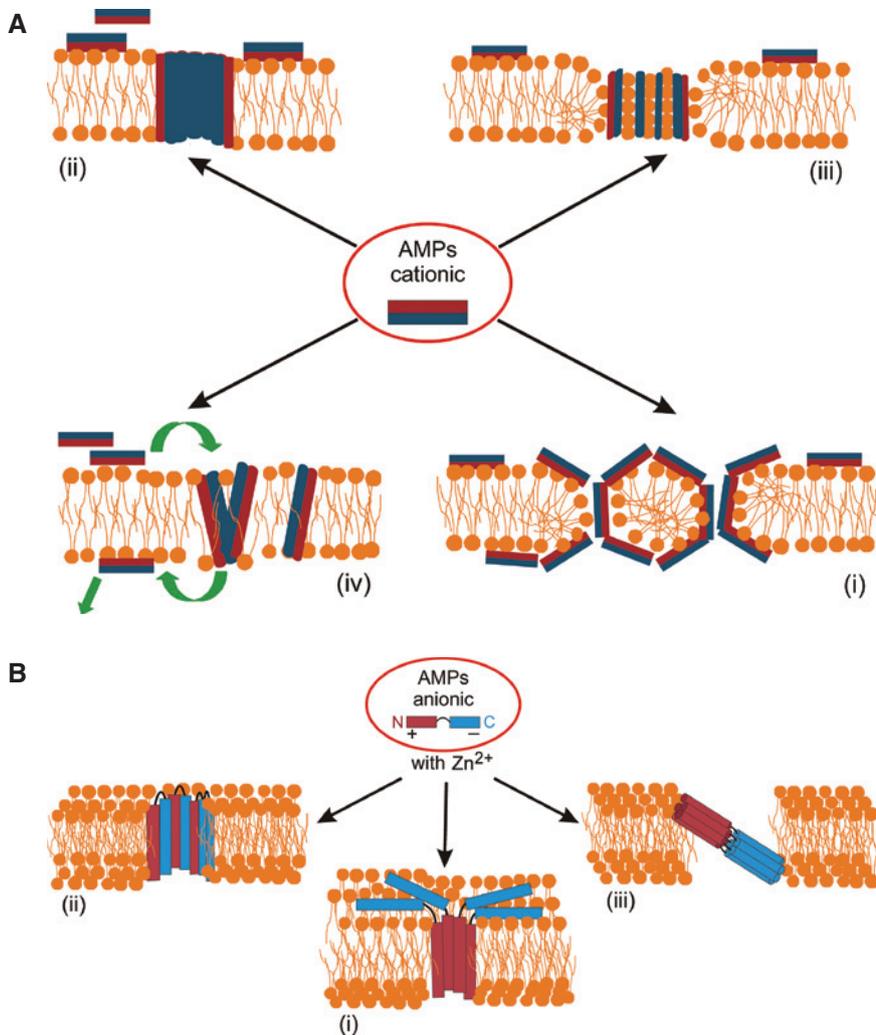
A number of defense peptides can traverse the cell membrane and enter the microbial cell, where they interact with nucleic acids, components of the translation apparatus, and protective proteins and cause disruption of replication, transcription, protein synthesis, and proper protein folding, which eventually results in cell

death (28, 29). The intracellular targets of some AMPs, e.g. anti-*Candida* histatins, are the mitochondria. Histatin localization to the mitochondria results in release of ATP, the extracellular activity of which is responsible for cell death (30). Non-helical AMPs have been demonstrated to interact with anionic ATP and directly inhibit the activity of ATP-dependent enzymes, thus interfering with the critical metabolic processes of a pathogen (31).

Most studies on the defense peptides-membrane interactions were performed using model lipid membranes, either composed of defined PLs or mimicking the composition of bacterial membranes. In contrast, although some mechanisms of the AMP antifungal action have been proposed (30), the interactions of defense peptides with fungal cell membranes have been much less investigated and therefore are less understood. One of the major differences between bacterial and fungal lipid membranes is the presence of ergosterol which, by causing a decrease in the membrane fluidity, can inhibit penetration of AMPs into the membrane as demonstrated for aurein 2.5, a frog  $\alpha$ -helical antifungal peptide (32, 33). Recent studies on plant antifungal defensins revealed their unique lipid binding profile specific for phosphatidylinositol-4,5-bisphosphate. Binding of the peptides with this PL involved the so-called ‘cationic-grid’ created by peptide dimers and led to formation of oligomeric complexes required for fungal membrane permeabilization. Interestingly, this mechanism has been suggested to be also involved in tumor cell lysis by plant defensin NaD1 from *Nicotiana glauca* (34, 35).

Some defense peptides, e.g. invertebrate members of the CS $\alpha\beta$  defensin family, human  $\alpha$ - and  $\beta$ -defensins, and  $\alpha$ -helical cathelicidins (LL-37) are cell wall active molecules. They inhibit peptidoglycan (PGN) synthesis by binding lipid II, which is responsible for delivery of the MurNAc-ppGlcNAc disaccharide precursor to the site of cell wall assembly. Human defensins and cathelicidins can also bind lipopolysaccharide (LPS), while some plant and insect defensins exert their antifungal activity via specific interactions with the sphingolipid components of fungal cell walls and membranes (36). Nevertheless, while cell wall synthesis and function may be disrupted by AMPs in some microorganisms, in others, the cell wall components may constitute important factors of pathogen resistance and can comprise a coat that protects against defense peptides.

It should also be mentioned that some defense peptides lack appreciable microbicidal activity and exert their protective action in a different way. One example of such a peptide is the human  $\alpha$ -defensin 6, which is highly expressed in secretory Paneth cells of the small intestine.



**Figure 1:** Interactions of cationic (A) and anionic (B) defense peptides with bacterial cell membrane.

(A) Four basic models of cationic AMPs interaction with a PL bilayer (orange) are presented. The cationic and hydrophobic domains are demonstrated in blue and red, respectively. The green arrows indicate possibility of traversing of a defense peptide through a microbial membrane into a cell. (B) Three proposed models of interaction of dermcidin-derived peptides as examples of anionic AMPs with a lipid membrane (orange) are shown. The cationic and anionic domains are colored red and blue, respectively. See text for further description.

Its antibacterial activity is expressed by binding to the bacterial cell surface, which induces ordered self-assembly of the defensin to form fibrils and so-called nanonets that surround and entrap bacteria (37).

### Interactions of anionic AMPs with microbial membranes

While the above-mentioned models of AMP binding with microbial cell membranes match well the typical cationic defense peptides, they do not explain how anionic AMPs interact with the usually anionic microbial cell surface. The amphipathic domains of such peptides and hydrophobic interactions are undoubtedly involved, but

are they strong enough to overcome the repulsive forces between the peptide and the cell surface? An evidence obtained in a study on the synthetic anionic peptide AP1, designed specially to adopt an oblique-oriented  $\alpha$ -helical conformation, revealed that its interaction with the bacterial membrane depends on the membrane lipid composition. In the interaction with lipid vesicles mimicking *Escherichia coli* membrane, AP1 used its hydrophobicity gradient to penetrate and destabilize the membrane acyl chains. In contrast, AP1 bound to the headgroup region of *Staphylococcus aureus* membrane thanks to positively charged lysylphosphatidylglycerol present in the membrane (38, 39). In this context, a recently published study on amphibian anionic maximin H5 should be mentioned, in which an important role of C-terminal amidation of this

peptide in its interaction with *S. aureus* membranes has been reported (40). This modification provides an additional positively charged moiety in the maximin 5 molecule, which facilitates targeting of anionic PLs in *S. aureus* membranes. In contrast, this modification has been proposed to underlie *E. coli* resistance against maximin 5 by forming hydrogen bonds with phosphatidylethanolamine headgroups in *E. coli* membranes. Such binding immobilizes the peptide molecules on the membrane surface, prevents penetration into the membrane, and finally inhibits the membranolytic action of the peptide (40, 41).

A detailed study on dermcidin-derived peptides DCD-1L and DCD-1, natural components of human sweat, revealed that they interact preferentially with negatively charged PLs, which is facilitated by the amphipathic structure of the peptides containing a cationic N-terminal and anionic C-terminal part. After an initial binding step when the cationic N-terminal region is electrostatically attracted by negatively charged PLs, the peptide eventually forms oligomeric channels stabilized by zinc ions in the membrane. Recently, three functional models and channel structures have been proposed: (i) the positive N-termini of the peptide molecules are embedded in the membrane with the negative C-termini floating on the surface, (ii) the cationic N-terminal regions of the peptides are folded back to the C-terminal regions forming transmembrane hairpins, and (iii) the peptides are tilted enough to maintain the channel completely in the membrane (Figure 1B) (23, 42, 43).

In the context of anionic defense peptides, it is important to note that neutralization of the cell surface negative charge is one of the resistance mechanisms evolved by bacteria, which prevents them from being killed by cationic AMPs (see the next section). One can hypothesize that anionic AMPs have evolved in response to such resistant microorganisms. However, when one takes into account the proposed models of interactions of  $\alpha$ -helical dermcidin-derived peptides with microbial membranes (23), which postulate an important role of the cationic N-terminus in initial binding, the emerging picture of the interactions between anionic AMPs and microbial cells appears to be much more complicated. On the other hand, recently Prabhu et al. (44) have characterized the first anionic defense peptide (isolated from plant *Cocos nucifera*) exhibiting antiproliferative activity against human glioma cell lines. An essential role in the initial interaction with the cancer cells has been ascribed to a short anionic sequence able to bind to positively charged PLs in the altered membrane of the cancer cells. To sum up, it seems that the interactions of anionic AMPs with their target cells are largely determined by the composition of

the target cell lipid membrane and presence of the particular PLs. However, still an unexplained question remains: how can anionic peptides bind to and traverse through the negatively charged barrier of the microbial cell wall?

## Mechanisms of microbial resistance to defense peptides

Due to the fact that the pathogens most likely co-evolved with AMP-producing hosts, they developed several resistance mechanisms against AMPs that are well documented in bacteria. Among others, the mechanisms include (i) sequestration of AMPs by charged components expressed on the surface or secreted by the pathogen, (ii) electrostatic repulsion through reduction of the surface negative charge by modification of cell wall components, (iii) changes in the composition of membrane PLs, (iv) efflux pumps, (v) degradation of AMPs by extracellular proteinases, and (vi) biofilm formation. They have recently been reviewed in excellent papers (45–50).

The discovery that some bacteria are able to sense AMP presence and in response appropriately change gene expression opened a new door to understanding the AMPs-pathogen interactions. Sensing of the AMP presence in an environment mediated by two-component signal regulatory systems has been reported in, e.g. *Salmonella enterica*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *S. aureus*. Activation of the systems PhoP-PhoQ, PmrA-PmrB, ParR-ParS, and CprR-CprS in Gram-negative bacteria and ApsS-ApsR and BraS-BraR in Gram-positive bacteria results in surface modifications that lead to reduction or neutralization of the negative charge, thus impeding interaction of cationic AMP with the cell surface (48, 51). What is threatening is that adaptive resistance to AMPs, similarly to aminoglycosides and a cationic antibiotic polymyxin B, has recently been reported. In *P. aeruginosa*, the CprR-CprS and ParR-ParS systems have been demonstrated to sense directly the cationic peptide indolicidin and synthetic AMPs. Moreover, exposure of *P. aeruginosa* cells to the human defense peptide LL-37 promoted synthesis of virulence factors and induced adaptive cross-resistance against aminoglycoside and fluoroquinolone antibiotics (52, 53). Importantly, AMP resistance in *P. aeruginosa* and *S. enterica* can be also induced by extracellular DNA (eDNA), which binds  $Mg^{2+}$  ions and activates PhoP-PhoQ and PmrA-PmrB two-component systems leading to protection against the AMP action (54, 55). Since eDNA plays an important role in the innate immune response as a component of neutrophil

extracellular traps which entrap invaders (56), the possibility of the above-mentioned scenario in an animal host cannot be excluded. Furthermore, Dobson et al. (57, 58) reported increased *in vivo* survival of experimentally selected iseganan-resistant *S. aureus* in an alternative insect model host. In addition, cross-resistance of pexiganan-resistant *S. aureus* to human  $\alpha$ -defensin, HNP-1, has been described (59). As iseganan and pexiganan are synthetic derivatives based on naturally occurring AMPs, porcine protegrin and frog magainin, respectively, one can hypothesize that the therapeutic use of defense peptide analogs carries risks of ‘arming the enemy’ with resistance not only to defense peptides involved in the immune response but also to designed synthetic AMPs.

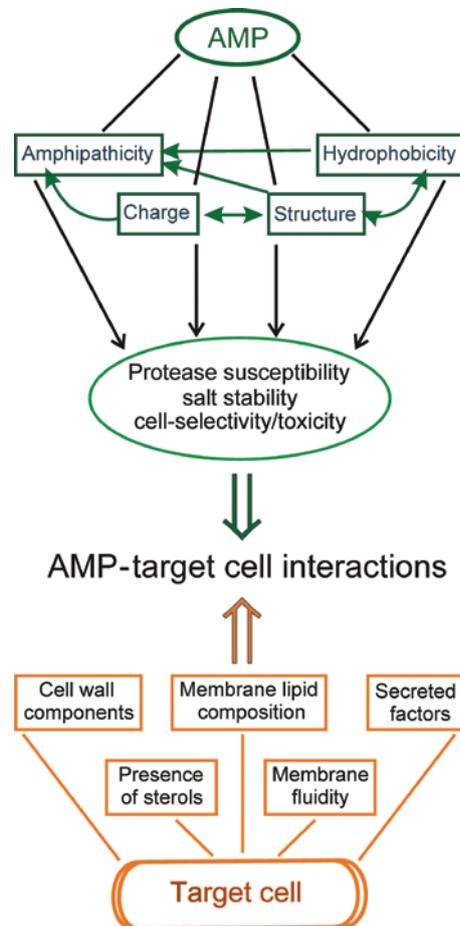
## Optimization and *de novo* design of AMPs

The design of novel AMPs necessitates optimization of multiple parameters, including peptide- and pathogen-based factors. Potential problems to be overcome include toxicity against host cells, salt instability, susceptibility to proteolytic degradation, development of allergies to the peptides, potential immunomodulatory activity, microbial resistance, and high production costs. Many structure-activity relationship studies using different natural and synthetic model peptides demonstrated that the potencies and spectra of the peptide antimicrobial activities can be influenced by alterations of the interrelated structural and physicochemical parameters, e.g. charge, hydrophobicity, and amphipathicity (60, 61). Realizing the tremendous complexity of AMP-pathogen interactions and multiplicity of factors affecting these interactions (Figure 2), only some approaches undertaken for the design of effective AMPs are shortly addressed below. Their common goal is to improve the peptide antimicrobial potency and cell selectivity as well as enhance the peptide stability in the salt environment and resistance to proteolysis (Figure 3).

## Improvement of antimicrobial potency and cell selectivity

### Charge and hydrophobicity balance

The net positive charge is considered a most critical parameter for defense peptide cell selectivity (62, 63). However, its increase over a defined threshold could dramatically increase hemolytic activity with no significant

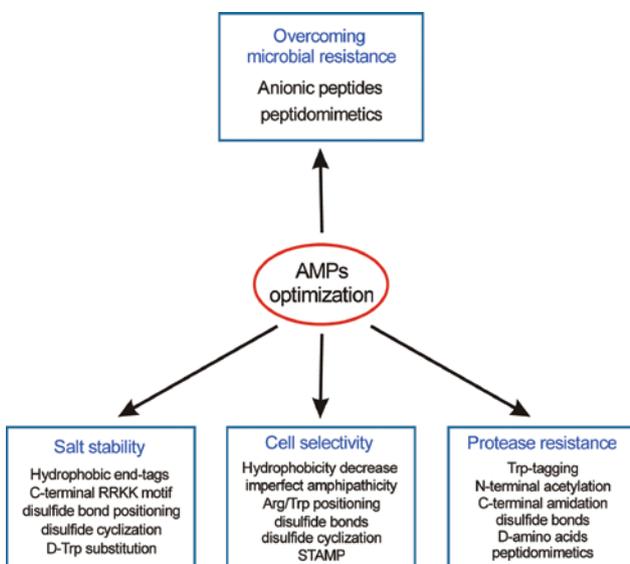


**Figure 2:** Scheme summarizing complex interactions between a defense peptide and a target cell.

Peptide-based and cell-based factors affecting the interactions are presented. An influence of the peptide properties on protease susceptibility, salt stability, and cell selectivity is indicated by the black arrows. A one-directional and mutual relationship between the essential properties of a peptide molecule is indicated, respectively, by the one- and bidirectional green arrows.

change in the antimicrobial potency as demonstrated by Jiang et al. (64) using designed analogs of the 26-residue amphipathic  $\alpha$ -helical peptide L-V13K. On the other hand, the hydrophobic nature of AMPs enables them to bind and insert into lipid membranes without electrostatic attraction. However, excessively hydrophobic peptides are indiscriminately toxic to eukaryotic and bacterial cells.

The proper balance between charge and hydrophobicity, necessary for the peptide maximum antimicrobial potency and minimum toxicity to host cells, can be achieved by introducing and/or replacement of important amino acids in defined positions of the peptide chain. In *de novo* designed AMPs, Arg and Lys are popular choices, as they are protonated under physiological conditions. Although both amino acids have comparable charge and



**Figure 3:** Selected ways to enhance therapeutic potential of AMPs. Approaches used to improve cell selectivity, salt stability, and resistance to proteolysis are summarized. Potential ways to overcome development of microbial resistance are also suggested. STAMP – specifically targeted antimicrobial peptide.

hydrophobicity, Lys primary amine interacts less electrostatically with PLs than the Arg guanidinium group, which could explain the stronger binding affinity of Arg-containing peptides to lipid membranes than that of Lys-containing counterparts (65–67).

One of the most extensively investigated designs for small synthetic AMPs is the combination of cationic Arg with hydrophobic Trp residues (65, 68–70). Due to its uncharged side chain, the Trp residue has a distinct preference for interaction in the interfacial region of lipid bilayers in comparison with Arg. The Arg residue gives the peptide a cationic charge and hydrogen-bonding properties necessary for interaction with the abundant anionic components of bacterial membranes (71). However, while replacement of all the Lys residues by Arg residues in the parent peptide PRW4 improved antimicrobial activity and cell selectivity of the designed peptides (72), the Arg-containing Trp-rich model peptides ( $R_6L_2W_3$ ) were more toxic to mammalian cells than their Lys-containing counterparts ( $K_6L_2W_3$ ) (73). The results of a study on  $R_6L_2W_3$  and  $K_6L_2W_3$  peptides indicate that the membrane and the cytoplasm of *Candida albicans* cells may be the ultimate target site for Arg- and Lys-containing peptides, respectively. Hence, even a slight change in the cationicity and hydrophobicity caused by replacement of important amino acids in the peptides can affect considerably their mode of action (73). Importantly, as demonstrated by Nagaoka et al. (74) using 18-mer peptides derived from human cathelicidin

LL-37, different functions of the peptide, e.g. antibacterial activity and LPS-neutralizing properties, can be concurrently influenced by proper alteration of hydrophobicity and cationicity.

An appropriate positioning of Trp residues at the hydrophobic-hydrophilic interface is important in order to enhance helical stability and increase the penetration of designed AMPs into microbial lipid bilayers with minimal effects on mammalian cell membranes. Yang et al. (75) showed that perfectly symmetric Trp-rich peptides ( $VRRF-PWWWPFLLRR-NH_2$ ;  $KKFPWWWPFKK-NH_2$ ) containing a WWW motif and a basic amino acid at the N- and/or C-terminus had strong antimicrobial activity against several antibiotic-resistant bacteria without hemolytic activity. Another example of promising Trp-rich peptides is the sequence  $XXWXXXWXXX-NH_2$  (X is K or L), which could be used to design 11 amino acid cationic Trp-rich AMPs, with different ratios of Lys and Leu (73, 76).

An interesting approach is a design of synthetic peptides with properly balanced cationic and hydrophobic content within the repeated units. Such a peptide can be simply composed of a repetition of the  $(XXX'X)_n$  amino acid sequence, where X is a hydrophobic amino acid, X' is a cationic amino acid, and 'n' is the number of repeat units. The study by Wiradharma et al. (77) revealed that the synthetic  $\alpha$ -helical AMPs with three repeat units,  $(FFRR)_3$ ,  $(LLRR)_3$ , and  $(LLKK)_3$ , were more selective towards microbial cells than red blood cells. In general, the peptides composed of two and three repeat units had no hemolytic activity, while those with four repeat units were highly hemolytic (>60%) even at low concentrations.

### Amphipathicity

Especially for  $\alpha$ -helical peptides, amphipathicity is a prerequisite for antimicrobial activity and has been considered the most important feature for the structural modification of natural peptides and design of synthetic peptides (62, 78). However, although this property contributes to strong antimicrobial activity, perfect amphipathicity has often been connected with increased hemolytic activity. Recently, an alternative approach to the design/optimization of AMPs has been suggested where imperfect amphipathicity of  $\alpha$ -helical peptides correlated with increased antimicrobial and reduced hemolytic activity. The amphipathicity of the helix could be disrupted by substitution of polar amino acids in the homogeneous hydrophobic face or hydrophobic amino acids in the cationic face and by replacing key amino acids with their D-enantiomers (79–81). In recent studies,

Zhu et al. (82) indicated that disruption of amphipathicity by replacing charged amino acids on the helix polar face with Trp endowed the AMPs with increased cell selectivity and antimicrobial activity against Gram-positive bacteria *S. aureus*, *S. epidermidis*, and *Streptococcus faecalis* and Gram-negative bacteria *E. coli* and *Salmonella Typhimurium*. In addition, the peptide with hydrogen bonds formed by paired Trp residues displayed stronger antibacterial and reduced hemolytic activity, indicating that the high antimicrobial potency and the concurrent low degree of hemolysis depended on a defined disruptive pattern. In further studies, Zhu et al. (72) demonstrated that the antimicrobial potential of amphipathic peptides could also be enhanced by D-Trp-substitution. The designed D-Trp-substituted peptide of imperfect amphipathicity showed greater antimicrobial potency in comparison with the L-Trp-substituted parent and maintained the activity in the presence of physiological salts and human serum. Interestingly, introduction of isoleucine (Ile; I), which slightly increased the  $\alpha$ -helix content in the peptide (otherwise known as a weak destabilizer of the  $\alpha$ -helix), or helix-breaker Pro into the peptide sequence compromised the peptide antibacterial activity, indicating that the propensity to form the  $\alpha$ -helix structure is not a prerequisite for strong antimicrobial activity. The investigated peptides had no cytotoxicity, which suggested that the subtle disruption of amphipathicity by introduction of paired amino acids on the polar face might be a method for designing low-cytotoxicity peptides. In contrast to the delicate balance of cationicity and hydrophobicity required for maximum antimicrobial potency and minimum toxicity to host cells, perfect amphipathicity and high propensity for  $\alpha$ -helix formation are unnecessary for achieving optimal activity and specificity of the designed peptides.

Disulfide bridges play a crucial role in the proper folding of diverse families of cysteine-rich AMPs. They stabilize the amphipathic topography of the peptide molecule, thereby maintaining its ability to interact with the target membranes and also confer protease resistance. Recently, Hwang et al. (83) have demonstrated that the introduction of intramolecular disulfide bridges to amphipathic cationic AMPs decreased their  $\alpha$ -helicity and hydrophobicity, which was accompanied by extreme reduction of their hemolytic activity. The mutant peptides have significantly increased (250-fold) the minimum hemolytic concentrations while maintaining the minimal inhibitory concentration values against *E. coli*.

The disulfide cyclization of the peptides can also considerably improve the antimicrobial activity and selectivity owing at least in part to increased amphipathicity and stability, with more distinct segregation of positive

and hydrophobic residues brought about by conformational constraints within the cyclized molecule. These parameters of cyclic peptides can be further modified and improved by the aromatic WXW motif (X=either W or F) (84, 85).

## Improvement of salt stability and protease resistance

### Salt stability

Many cationic AMPs such as indolicidins, magainins, bactenecins, and defensins partially or completely lose antimicrobial activity at physiological salt concentrations of approximately 150 mM NaCl, 1 mM Mg<sup>2+</sup>, and 2 mM Ca<sup>2+</sup> (65). High salt concentrations can reduce electrostatic adsorption of cationic peptides onto the anionic microbial cell surface. Binding of cationic AMPs to Gram-negative bacteria is additionally impeded by the divalent cations such as Ca<sup>2+</sup> and Mg<sup>2+</sup> that compete with the peptides for binding with LPS in the bacteria's outer membrane.

It has been demonstrated that the C-terminus RRKK motif of human  $\beta$ -defensin-3 (HBD-3), one of the few salt-tolerant AMPs, contributes to its salt-resistant behavior. The cationic RRKK motif probably overcomes salt intolerance by reducing charge-shielding effects, which cause inactivation of many AMPs (86, 87). Li et al. (88) engineered a mutant HBD-28 with the C-terminus RRKK, yielding a high-salt-tolerant peptide with broad-spectrum antibacterial activity, which confirmed the important role of this motif in establishment of salt stability. The results of Saravanan et al. (70) indicated that the RRKK motif combined with RW residues confers potential activity of RW-rich decamers engineered from a salt-resistant human HBD-28 variant against pathogenic bacteria (*E. coli*, *P. aeruginosa*, and *S. aureus*) in the presence of salt by considerable improvement of selectivity towards the bacterial cell membrane.

Another approach to improve the antimicrobial activity and salt stability of the peptides is end-conjugation with hydrophobic moieties. The choice of Trp as hydrophobic end-tags is attractive as the bulky aromatic ring has high membrane interfacial affinity that facilitates deeper penetration of the membranes by the peptides. Pasupuleti et al. (89) demonstrated that, when tagged with WWWW, kininogen-derived short peptides had bactericidal potency under high ionic strength (150 mM NaCl) and limited toxicity compared to the non-tagged peptides. The enhanced antimicrobial activity of W-tagged AMPs was accompanied by stronger binding

of the peptides with LPS and selective permeabilization of the bacterial membrane. On the other hand, Chu et al. (90) found that the end-tagging of short Trp-rich AMPs (KWWK and Ac-KKWRKWLAKK-NH<sub>2</sub>) with a single amino acid  $\beta$ -naphthylalanine was sufficient for their protease resistance and effectiveness in the presence of salt. When Yu et al. (91) replaced Trp or His residues in the histatin derivative P-113 with  $\beta$ -naphthylalanine and  $\beta$ -(4,4'-biphenyl)alanine, the analogs retained antimicrobial properties under high-salt conditions up to 300 mM NaCl or 2.5 mM MgCl<sub>2</sub>. Kim et al. (92) designed seven  $\alpha$ -helical peptides with hydrophobicity from 33% to 46% and positive charge from +4 to +5 and found that increasing the hydrophobicity of the peptides by replacement of all Val residues with Ile or Leu residues maintained their activity in the presence of 150 mM NaCl.

Salt tolerance of AMPs can also be enhanced by stabilization of conformational constraints through dimerization or cyclization via properly positioned disulfide linkages (65). As demonstrated by Nan and Shin (93) using a dimeric model peptide di-K<sub>6</sub>L<sub>4</sub>W<sub>1</sub> (KLKKLWKKLLK-NH<sub>2</sub>)<sub>2</sub>, greater salt resistance may be achieved by location of a disulfide bond at the N- or C-terminus of the peptide dimers.

### Protease resistance

Susceptibility to degradation by endogenous human proteases and proteases secreted by invading pathogens is likely to be the most important cause of the poor *in vivo* activity of AMPs. Among the human proteases, trypsin and chymotrypsin are the greatest threats to AMPs. Trypsin and chymotrypsin attack peptides at basic (Lys and Arg) and hydrophobic residues (Trp and Phe), respectively, which is an obligate feature of AMPs. Peptides can be protected from degradation by, e.g. cyclization, modification of their terminal regions (N-terminal acetylation or C-terminal amidation), or by incorporation of non-natural amino acids (mainly D-form amino acids) (61, 65). Nevertheless, Kim et al. (92) succeeded in designing two peptides GNU6 (RIIRPIIQIKQKIR) and GNU7 (RLLRPLLQLLKQKLR) consisting of unmodified natural amino acids, which were not digested when exposed to trypsin, chymotrypsin, and aureolysin for up to 12 h and showed potent antimicrobial activity against methicillin-resistant *S. aureus* and vancomycin-resistant enterococci. Moreover, they did not affect the viability of erythrocytes, keratinocytes, and fibroblasts.

The advantage to the stability of AMPs in biological milieus is cyclization through an intramolecular

head-to-tail backbone amide linkage, disulfide bridging, or native chemical ligation (84, 94, 95). The conformational constraints introduced with the cyclization of AMPs are believed to enhance protease stability due to the reduced accessibility of the mobile ends of the peptide chains to protease binding and cleavage, as demonstrated for, e.g. an indolicidin analog CP-11 and Trp- and Arg-rich hexapeptides based on the lactoferrin 'antimicrobial center' sequence. The terminal modification of the lactoferrin-derived hexapeptides enhanced their stability in the following order: both N-acetylation and C-amidation > N-acetylation > C-amidation (84). In addition, Stromstedt et al. (96) showed that the protease stability of EFK17, a LL-37-derived peptide, was enhanced when both N-acetylation and C-amidation were combined with Trp substitutions. Wang et al. (76) designed and synthesized an ideal amphipathic  $\alpha$ -helical Leu/Lys-rich model peptide K<sub>9</sub>L<sub>8</sub>W and its diastereometric analogs containing a different number and distribution of D-amino acids. The results suggested that a 33% D-amino acid substitution in cationic peptides is necessary to achieve complete resistance to enzymatic degradation by trypsin. It was also found that the end-tagging of short cationic peptides with one to five hydrophobic amino acids such as Trp, Phe, and  $\beta$ -naphthylalanine, particularly the Trp-tagging at the C-terminus, enhanced the protease stability in the presence of serum and increased the antimicrobial potency. Malmsten et al. (97) demonstrated that C-terminal end-tagging by Trp or Phe residues of the peptide sequences GRRPRPRPRP (GRP10) and RRPRPRPRP (RRP9) increased their antimicrobial activity. The 4W-tagged peptide RRP9WWWW-NH<sub>2</sub> had very high bactericidal activity, displayed no lytic activity and low toxicity against mammalian cells, and was highly resistant to the proteolytic degradation. The W-tagged peptides displayed stability against elastase and aureolysin and were effective against vancomycin-resistant enterococci, multidrug-resistant *P. aeruginosa*, and methicillin-resistant *S. aureus* (97).

The susceptibility of AMPs to proteolytic degradation may be circumvented by the use of antimicrobial peptidomimetics that contain non-peptidic backbones. Several classes of such molecules have been designed, e.g. AApeptides,  $\beta$ -peptides, peptoids,  $\beta$ -turn mimetics, and methacrylate copolymers. In addition to the broad-spectrum antibacterial activity, including methicillin-resistant *S. aureus* and multidrug-resistant *P. aeruginosa*, they can exhibit antiviral and antitumor activity, and some of them are currently being tested in clinical trials (98–100). Similarly to AMPs, such properties as cationicity and hydrophobicity of these synthetic molecules can be appropriately modified in order to obtain peptidomimetics with

desired activity. Recently, a human defensin mimetic that binds lipid II involved in bacterial PGN synthesis has been designed and demonstrated to be protective in an *in vivo* murine model of sepsis caused by *S. aureus* (101). Furthermore, Ryan et al. (102) developed nonpeptide mimetics, which were highly active and selective against *C. albicans* when tested in mouse models of oral candidiasis. As demonstrated for methacrylate copolymers, at least this class of peptidomimetics does not induce development of resistance in *E. coli* (100). However, taking into consideration the great flexibility and adaptability of microorganisms, a possibility of development of resistance to peptidomimetics in other pathogenic bacteria in the future cannot be excluded.

### Natural AMP-derived fragments and design of hybrid peptides

In order to reduce the costs associated with synthesis of AMPs, there have been attempts to identify truncated variants of peptides or minimal peptide units that retained the biological activities of the full-length peptide. The analysis of a synthetic peptide library derived from LL-37 (from 12 to 30 amino acid) showed the VQRIKDFLRN sequence to be critical for high antimicrobial activity (103, 104). Similarly, the ATRA-1 peptide, an 11-residue motif from the Chinese cobra (*Naja atra*) cathelicidin, had as high antimicrobial activity against Gram-negative bacteria as the parent peptide (105, 106). Haversen et al. (107) showed that a 14-amino acid fragment from the N-terminus of human lactoferrin was highly active against *E. coli*, *S. aureus*, and *C. albicans*. Interestingly, it was found that the optimal length for the antimicrobial activity of the lactoferrin-derived sequence is 12 aa (amino acids 19–31), but 13 aa (amino acids 17–30) was optimal for amebicidal properties (108–110). Recently, a new strategy to identify putative AMPs encrypted in protein sequences has been described. In this approach, candidate peptides were identified by *in silico* screening of protein databases on the basis of desired physicochemical criteria (111).

Designing hybrids of different AMPs or their fragments constitutes another approach for obtaining novel AMPs that combine the desirable properties of the individual parent peptides. The best-known example is the frequently studied hybrid peptide cecropin A-melittin (CAM), which exhibits a stronger and wider range of antimicrobial activity with less hemolytic activity than the individual parent peptides (112, 113). The CAM-W peptide obtained by replacement of four amino acid residues by Trp in the parent peptide had improved proteolytic stability, 3–12

times higher activity against drug-resistant bacteria, and strong activity against gastroenteritis-associated fungi (e.g. *Aspergillus flavus* and *C. albicans*) (114).

Several research groups have tested combinations of cecropin and melittin with other peptides or designed hybrids with new peptides (65, 115, 116). For example, Fox et al. (115) designed a novel hybrid AMP from fragments of insect cecropin A (CA), frog magainin 2, and human cathelicidin LL-37, using *in silico* design and modeling. Hybrid peptides with the best amphipathic cationic  $\alpha$ -helices were shown to have greater antimicrobial effects than those of the parent AMPs. Liu et al. (116) designed  $\beta$ -hairpin-forming hybrid peptides containing the 1–16 or 5–17  $\beta$ -fold regions of protegrin-1 (PG-1) with either the N-terminal cationic amphipathic 1–8 region of CA or the active 4–9 center of bovine lactoferricin in efforts to reduce the cytotoxicity of PG-1 towards eukaryotic cells. They showed that the hybrid peptides LB-PG and CA-PG exhibited broader spectrum of antibacterial activity and enhanced protease stability as well as improved hemolysis and cytotoxicity profiles, compared to the respective parent peptides. Wu et al. (117) obtained a hybrid peptide by combining a hydrophobic N-terminal fragment of melittin with a core antibacterial fragment of LL-37. Their results showed that the hybrid peptide had more potent antibacterial activity against all tested strains (especially Gram-positive bacteria) than melittin and LL-37 individually but did not exhibit hemolytic activity to sheep erythrocytes.

In regard to the hybrid AMPs design, a novel technology based on specifically targeted antimicrobial peptides (STAMPs) developed for controlling the cariogenic pathogen *Streptococcus mutans* in oral microbial communities should be mentioned (118, 119). A STAMP is a synthetic peptide that contains a pathogen species-specific peptide sequence and a non-specific AMP sequence, which function as targeting and killing domains, respectively. Such an approach allows selective elimination of a particular bacterial species from a multi-species community.

### Potential applications of antimicrobial peptides

The most attractive application of AMPs is their use as therapeutic alternative of conventional antibiotics. Examples of AMP-derived molecules that have been tested clinically include hLF(1-11), a human lactoferrin derivative (108, 120); pexiganan, a magainin 2 analog (121); iseganan, a protegrin 1 derived peptide (122, 123); omiganan, an indolicidin analog (124, 125); and the anti-*S. mutans* STAMP peptide C16G2 (118).

The use of AMPs in combinations with antibiotics may have considerable advantages in concurrent enhancement of antimicrobial potency and reduction of toxicity. Possibly, the synergistic action could minimize the required dose of therapeutics in comparison to the single molecule and prevent development of drug resistance in bacteria. Tests carried out *in vitro* and *in vivo* by Rishi et al. (126) demonstrated increased synergistic killing of intracellular *S. Typhimurium* by cryptdin 2 (Paneth cell AMP) with ampicillin, compared to the individual compound. Interestingly, the research showed reduction of the colony count of *S. Typhimurium* in the target organs of mice (liver, spleen, and small intestine) after subcutaneous injection of cryptdin 2 with ampicillin together at lower doses than those of the individual agents. The study conducted by Anantharaman et al. (127) showed that a combination of four designed peptides, which had poor to moderate antimicrobial activity, with rifampicin or kanamycin enhanced their potencies against *E. coli* 4- to 34-fold, respectively. Synergistic effects were reported for the lactoferrin-derived peptide hLF(1-11) and fluconazole against *C. albicans*. It was also observed that lactoferrin or hLF(1-11) added in subinhibitory concentrations to antifungal agents such as clotrimazole, ketoconazole, and itraconazole reduced the minimum inhibitory concentration of these agents against *Candida* species (108, 128, 129).

An alternative idea of using AMPs is application thereof in prevention of infections as coating agents for medical devices, dental or bone implants, or in biosensing applications (108). Covalent immobilization of AMPs onto a biomaterial surface through different chemical coupling strategies offers many advantages by overcoming potential limitations, such as short half-life and cytotoxicity associated with higher concentrations of soluble peptides. This could be effective in the prevention of biofilm formation by reduction of microorganism survival post-contact with coated biomaterial (130, 131). Recently, Di Luca et al. (132) reported on construction of BaAMPs, a new database collecting biofilm-active AMPs.

AMPs may also have potential application in the control of agricultural diseases by engineering them in plants to confer resistance to phytopathogens (133). For example, plant defensins expressed in transgenic rice (134), wheat (135), and banana (136) protected the plants against fungal pathogens. Similarly, transgenic expression of an insect defensin, *G. mellonella* gallerimycin, and cecropin, *Sarcophaga peregrina* sarcotoxin-IA, in tobacco also conferred resistance to pathogenic fungi (137, 138). Transgenic expression of *Drosophila melanogaster* metchnikowin protected barley against ascomycete fungal

pathogens, indicating that insect antifungal peptides are a valuable source for crop plant improvements (139). Interestingly, co-expression of insect AMPs, cecropin and defensin, in transgenic *Aedes aegypti* infected with *Plasmodium gallinaceum* dramatically reduced the parasite development and completely blocked transmission thereof (12). Since one of the possible approaches for controlling disease transmission is population replacement with genetically modified vectors, transgenic mosquitoes overexpressing cooperatively acting AMPs constitute a promising opportunity.

## Outlook

The great diversity of defense peptides evolved and offered by Nature indicates that obviously there is no one universal 'gold antimicrobial peptide' and there is no one solution in the fight with microbial pathogens. This diversity and simultaneous expression of different defense peptides by a given organism also reflects a necessity of protection against development of microbial resistance to AMPs. Despite the early hopes that pathogens would not easily develop such resistance, now it is clear that some bacteria already have. In this regard, further detailed studies on the structure, properties, and interactions of promising anionic defense peptides and peptidomimetics with model microbial membranes and whole pathogen cells could provide valuable information.

Nowadays, the diversity of defense peptides might be substantially extended by improving molecules occurring in nature and by creating new ones. Modern computational approaches in alignment with *in silico* models, advanced peptide microarrays allowing identification of potent AMPs, and novel isolation techniques together provide tools for the design and optimization of novel effective molecules with desired properties. For improving their activity in a defined direction, detailed understanding of the molecular mechanism of action of a particular AMP but also prediction of potential resistance mechanisms are a very important issue.

In the design of AMPs with therapeutic potential, numerous peptide-based as well as pathogen-based factors are taken into account. Nevertheless, due to the lack of well-defined and unambiguously defined mechanism of action and the complexity of the target cells, elaboration of general rules used for AMP design is almost impossible. The low toxicity to eukaryotic cells, high salt tolerance, and resistance to proteolytic digestion are undoubtedly critical parameters of a therapeutic

AMP. From the economic point of view, especially the broad-spectrum activity is a great advantage. However, this poses a danger of elimination of beneficial microflora from an organism, a well-known consequence of treatment with conventional antibiotics, as non-pathogenic microorganisms most probably are more susceptible than the pathogenic ones. Taking this into account, the STAMP technology mentioned in the review, although unquestionably more cost consuming, seems to offer a greater level of safety by designing very species-specific peptides, which facilitate elimination of a particular pathogen from a multi-species microbial community.

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## List of abbreviations

AMP	antimicrobial peptide
LPS	lipopolysaccharide
PGN	peptidoglycan
PL	phospholipid
STAMP	specifically targeted antimicrobial peptide

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