

Concerns in the Design and Development of Novel Antimicrobial Peptides

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HIGHLIGHTS

- Antimicrobial peptides are highly interesting antibiotics in multi-drug resistance infections.
- Antimicrobial peptides are short peptides with less than 50 amino acids and overall positive charge.
- First and second structural features of AMPs are important factors in their bioactivities.
- Cyclization and branching of AMPs could affect their pharmacokinetic and pharmacodynamics.

ABSTRACT

Peptide and protein based therapeutics are the most promising approaches in today medicine. Bioactive peptides can be valuable drugs in the treatment of various illnesses, such as cardiovascular and neurodegenerative diseases. Cell toxic peptides can be considered for cancer or infection therapy. Antimicrobial peptides (AMPs) are one of the most interesting antibiotic groups in this regard, especially in drug resistance infections. Numerous AMPs have been discovered from the natural source; however, artificial synthetic ones have been also developed based on rational design or bioinformatics modeling. Physicochemical features of AMPs are highly important in their antibacterial activity as well as their toxicity. The best AMP is the one that has selective potent antimicrobial bioactivity and no or least hemolytic and cytotoxic effect. In this review, various structural factors affecting the AMPs bioactivity, such as AMPs size, charge, amphipathicity, and amino acid sequence are illustrated considering the most recently published articles. Finally, the trends in AMP design and development are discussed.

Keywords:

Activity improvement
Antimicrobial peptide
Computational tools
Rational design

Introduction

Nowadays, multi-resistant bacterial strains are the most challenging issue in the treatment of infections. Several important species of the resistant bacteria are methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-

resistant *Enterococci*, carbapenem-resistant *Klebsiella pneumoniae*, resistant species of *Acinetobacter baumannii*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Clostridium difficile*, and *Mycobacterium tuberculosis* (Zaslhoff et al., 2011). The emergence of this bacterial resistance has encouraged the search for novel antibiotics with improved action against hard to treat bacterial infections. Antimicrobial peptides (AMPs) as an evolutionarily conserved component of the innate immune response in living organisms are found to be an efficient solution for

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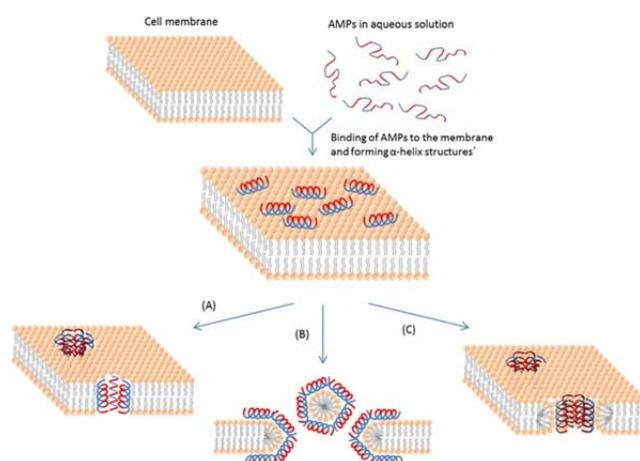


Figure 1. Schematic representation of membrane activity as the main mechanisms for positively charged AMPs. (A) Barrel-Stave model. Vertically insertion of AMP molecules into the membrane. (B) Carpet model. AMP molecules surrounded small areas of the membrane by hydrophobic sides facing introducing pores in the membrane. (C) Toroidal pore model. Like the Barrel-stave model, peptides are assumed to adopt a trans-membrane orientation but AMPs are always in contact with phospholipid head groups. The blue and the red colors represent the hydrophobic and hydrophilic portions of AMPs, respectively (Bahar and Ren, 2013).

resistant bacterial infections (Giuliani et al., 2007). These nonspecific ancient defense agents are simple, rapid, and multifunctional mediators (Gordon et al., 2005). Various sequences of human natural AMPs originated from different cells/tissues such as cathelicidin LL-37 from the skin of new born infants, α -Defensin from neutrophils, and Histatin from saliva have been discovered so far (Wang, 2014).

Structure and amino acid sequences of AMPs have a wide diversity (Table 1), but as a general definition, AMPs are short peptides with less than 50 amino acids and overall positive charge, which include hydrophobic amino acids in balancing with charged part (Hancock, 2001). Regarding these properties, AMPs fold into amphipathic conformation upon contact with negatively charged bacterial membranes (Fig. 1). Traditional antibiotics generally interact with specific intracellular targets. Nevertheless, AMPs physically interact with the bacterial membrane and rapidly destroy it, then permeate to the cells and can destroy vital components inside the cell. Consequently, there are very low chances of developing AMP resistance by alteration of the membrane. Most AMPs display broad activity against both gram-positive and gram negative bacteria. Important challenges in therapeutic applications of AMPs are their low stability, relatively high toxicity, and high manufacturing cost (Kim et al., 2014). Some of these cationic peptides exhibit anti-viral (Currie et al., 2016; Novoa et al., 2016; Methatham et al., 2017), anti-inflammatory (Brook et al., 2016; Lee et al., 2016), immune-modulatory (Hancock et al., 2016; van

Dijk et al., 2016) and wound healing activities (Mangoni et al., 2016; Sørensen, 2016) in addition to antimicrobial activity.

Methods applied for new antimicrobial peptide design

Besides the discovery from natural sources, novel AMPs can be developed following a rational *de novo* design (Lashua et al., 2016) or mutation/conjugation approaches on the previously established AMP sequences (Haney et al., 2012; Jung et al., 2011) to improve specific antimicrobial activity and therapeutic index.

Bioinformatics tools

Peptides with different amino acid sequences, structures, and bioactivity exhibit various important roles in biological systems. Application of computer-based techniques for *in silico* design or development of novel more effective therapeutics has been progressed in the fields of peptides and proteins. Databases of peptides or proteins are the practical valuable computational tools for new drug design, composed of peptide sequences and structures with different bioactivities. There are different antimicrobial databases that could be applied for new peptide design (<https://omictools.com/antimicrobial-peptide-data-category>). One of them is the Antimicrobial Peptide Database (APD) that is a major database of natural peptides with antimicrobial activity. The APD (<http://aps>.

Table 1. Examples of AMPs with different length and source.

Name	Source	Sequence	length	Biological activity	Ref.
SCY2 (Seygonadin)	<i>Scylla paramamosain</i>	GLALNRLMKNKAVDAIVVMVGQQDAGVSL GHPCLVESAKQPEGIYAVMSCASWTPRFV EGTSEVELEALKGSIKRSFKKASDYQLLSKED LEDWLASY	100	Antibacterial (Anti-gram positive bacteria) Could exert reproductive immunity and maintain sterility in the spermatheca	(Qiao et al., 2016)
PRD- SpHyastatin	Designed based on SpHyastatin form <i>Scylla paramamosain</i>	YNAKVPIQTLPERLDNFPGRGPFTRPAVGV QTLPGRVPPQTFPGVIGVTKPLISPPRPGFTG STRPFQRPQGQYSFTR	80	Antimicrobial activities against the human pathogen and the aquatic animal pathogens	(Shan et al., 2016)
ToAMP2	<i>Taraxacum officinale</i> flowers	GGKCTVDWGGGGRRRLPSLFCCKYKPTRIC YLNQETCETETCP	44	Active against phyto-pathogenic fungi and bacteria	(Astafieva et al., 2012)
ToAMP3	<i>Taraxacum officinale</i> flowers	ANKCIIDCMKVKTTTCGDECKGAGFTGGCA LPPDIMKCCCHNC	42	Active against phyto-pathogenic fungi and bacteria	(Astafieva et al., 2012)
ToAMP1	<i>Taraxacum officinale</i> flowers	VAKCTEESGGKYFVFCCKYKPTRICYMNEOK CESTCIGK	38	Active against phyto-pathogenic fungi and bacteria	(Astafieva et al., 2012)
CRD -SpHyastatin	Designed based on SpHyastatin from <i>Scylla paramamosain</i>	SNCWARCPGYPNGDSLCCROYGACSTSY PVPYKG	35	Antimicrobial activities against the human pathogen and the aquatic animal pathogens with improved activity compared to Proline rich domain (PRD-SpHyastatin)	(Shan et al., 2016)
AG-30	Human cDNA library and <i>in silico</i> analysis	MLSILIFLHRLKSMRKRDRKRLRWRKKNYP	30	Antimicrobial activity against <i>P. aeruginosa</i> , <i>E. coli</i> , and <i>S. aureus</i> Angiogenic activity	(Nishikawa et al., 2009)
Thaulin-1	Patagonian frog <i>Pleurodema thaul</i>	NGNLLGLLRPVLGVVKGGLTGGLGKK	26	Moderate antimicrobial activity against <i>E. coli</i>	(Marani et al., 2017)
PvHct	<i>Litopenaeus vannamei</i>	FEDLPNFGHIQVKVFNHGHEHHH	23	Antimicrobial activity Antifungal activity	(Petit et al., 2016)
N2	Designed based on NZ17074 from <i>Arenicola marina</i>	(C-terminal of hemocyanin)	21	Antimicrobial activity against gram negative bacteria	(Yang et al., 2016)
Hepcidin 25 (LEAP-1)	Human plasma, Urine/Liver	AFCWNVCVYRNARVYCHRRCN	20-25	Antifungal activity Antibacterial activity against gram negative and gram positive species	(Park et al., 2001)
ApoE (133-150)	Human Apolipoprotein E	LRVRLASHLRKLRKRLLR	18	Antimicrobial and anti-inflammatory/ Immunomodulatory activities	(Pane et al., 2016)
T9W	<i>De novo</i> design	RFRRRLKRWKRLKLI-NH ₂	16	Antimicrobial activity, specifically against <i>Pseudomonas aeruginosa</i>	(Zhu et al., 2014)

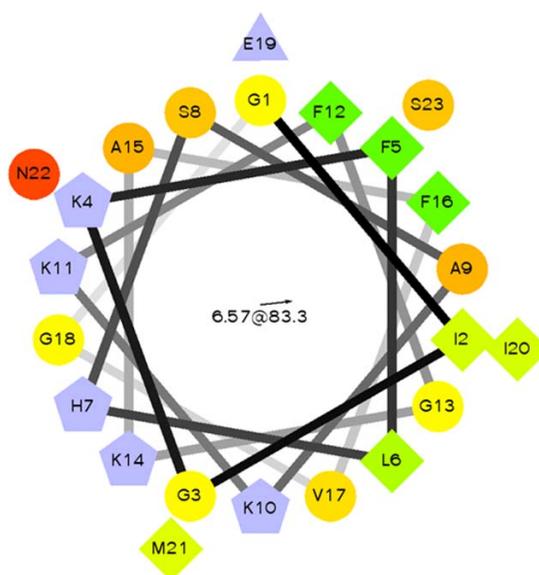


Figure 2. Helical Wheel presentation of Magainin II. By default, the hydrophilic residues are presented as circles, hydrophobic residues as diamonds, potentially negatively charged as triangles, and potentially positively charged as pentagons. The most hydrophobic residue is green, and the amount of green is decreasing proportionally to the hydrophobicity, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red with pure red being the most hydrophilic (uncharged) residue, and the amount of red decreasing proportionally to the hydrophilicity. The potentially charged residues are light blue.

unmc.edu/AP) can facilitate impressive search, analysis, design of antimicrobial peptides and, so on (Wang, 2015). The other is the Collection of Anti-Microbial Peptides (CAMP) that is a database of sequences, structures, and family-specific signatures of prokaryotic and eukaryotic AMPs (<http://www.camp3.bicnirrh.res.in/index.php>). More than 4000 AMPs collected in this dataset presented 10247 sequences, 757 structures and 114 family-specific signatures of AMPs (Waghu et al., 2015). Moreover, DADP (Database of Anuran Defense Peptides) is another antimicrobial database (<http://split4.pmfst.hr/dadp/>), which currently contains 2571 entries. For some of the peptides, the minimum inhibiting concentration (MIC) against at least one microorganism was accessible (Novković et al., 2012).

Among computational approaches for design novel antimicrobial peptides, different strategies of structure-activity relationship analysis could be considered (Blondelle and Lohner, 2010; Alvarez-Ordóñez et al., 2013; Abraham et al., 2014). According to the results of such studies, some webserver provide the users with an ability to evaluate new peptide sequences as an antimicrobial agent. The dPABBs (Design Peptides against Bacterial Biofilms) webserver (<http://ab-openlab.csir.res.in/abp/antibiofilm/index.php>) facilitates the prediction and design of anti-biofilm peptides. A

prediction strategy was developed for the identification and optimization of novel anti-biofilm peptides providing features like simultaneous multi-model predictions and mutant generation (Sharma et al., 2016).

In addition to the biological activity, several other online free services provide the structural and conformational information. As the alpha helical structure of cationic AMPs and their amphipathicity are two of the important features of these peptides, the scientists have evaluated the alpha helix formation and the positions of hydrophobic and hydrophilic residues by Helical Wheel Projections from RZ Lab (<http://rzlab.ucr.edu/scripts/wheel/wheel.cgi?submit>). Helical wheel is a type of visual representation to illustrate the characteristics of alpha helices in peptides and proteins (Fig. 2) (Juretić et al., 2017; Pedron et al., 2017).

Rational design

Rational design of AMPs includes considering different prominent physicochemical aspects of formerly established AMPs to design and develop new peptides with improved antimicrobial and decreased undesirable properties like hemolytic activity and human cell cytotoxicity. The antimicrobial properties of AMPs depend on multiple features including their size, amino

acid sequence, hydrophobicity of amino acid side-chains, the positive charge distribution, and the structural conformation induced through the contact with the bacterial membrane (Fjell et al., 2010; Fjell et al., 2012).

One of the strategies applied for designing novel AMPs is that one natural AMP sequence is considered as the initiation template, and then, systematic sequence truncation, amino acids substitution, and cyclization are evaluated to obtain an improved antimicrobial therapeutic agent. The other strategy is the study of the structure–activity relationship (SAR), which could help scientists improve antimicrobial activity and selectivity (Ong et al., 2014). Using analytical approach, a research group designed a novel peptide sequence using the genetic algorithm. This algorithm considered the values of net charge, hydrophobicity, isoelectric point, and instability index within the specified user-established ranges to introduce a new antimicrobial peptide sequence. They designed a 17 amino acids peptide that showed promising MIC value for *E. coli* and MRSA with no hemolytic activity in these concentrations. Therefore, it would be a potential antibiotic instead of the existing ones in clinical use (Prada et al., 2016).

Features considered in antimicrobial peptide design

Length

Primary sequence of AMPs can affect the antimicrobial as well as hemolytic activity. Studies showed that AMPs with smaller length are less hemolytic. Several researchers studied how the elimination of some unessential parts of peptide sequences affect the toxicity and immunogenicity of AMPs. For instance, Zaslouf et al. showed that omitting the three amino acids (Gly-Ile-Gly) from N-terminus of magainin 2 would not adversely affect the antimicrobial activities, but elimination of four or more residues would decrease the activity (Ong et al., 2014). In the design of new AMP sequences, the length of about 10 amino acids or less has been more promising than the longer sequences, according to the biological activity and toxicity (López-García et al., 2002). Generally, short length antimicrobial peptides (natural or synthetic) are the most exciting ones in the pharmaceutical development, as the cost of production decreased and in most of cases less toxicity have been reported (Domalaon et al., 2016).

Charge

As a rule, positively charged residues lead to the AMPs accumulation around the pathogens, through electrostatic interactions with membrane anionic phosphate groups. After achieving a critical concentration, AMPs insert to the lipid membrane by the hydrophobic part of their amphiphilic structure and result in membrane disruptions

(Lee et al., 2016).

Positive charge of antimicrobial peptides is one of the important features of AMPs affecting their bioactivity. Presence of Arg and Lys as the positive amino acids interacting with high negatively charged microbial membrane enhanced the antimicrobial activity. Therefore, these positive residues are the rational structural components in the design of novel AMPs influenced their antimicrobial activity and selectivity (Ong et al., 2014).

In a study on AR-23 as a melittin-related peptide with 23 residues, Zhang et al. evaluated the substitution of two Alanine residues and one Isoleucine residue in different parts of peptide, with Arg or Lys as the positively charged amino acids. They studied the effect of positive charge distribution on the antimicrobial and hemolytic activity of designed peptides. Lys substitution of Ile17 on the nonpolar face significantly reduced the hydrophobicity, amphiphaticity, and therefore, reduced the human cells cytotoxicity and hemolytic activity of the peptide. However, substitution on the polar face slightly affected the peptide biophysical properties and biological activity. As a result, the number and position of positively charged residue could have effect on the biophysical properties and selectivity of the biological activity. Several of these designed peptides showed better therapeutic index as an antimicrobial agent for the reason that they exhibited similar antibacterial activity with lower cytotoxic and hemolytic effect compared to the parent peptide (Zhang et al., 2016).

One promising example of the synthetic AMPs is BP100 with a short sequence of KKLFFKKILKYL-NH₂ discovered through combinatorial chemistry approach. The first design was based on the cecropin A and melittin as natural AMPs. BP100 is a potent antimicrobial agent against Gram-negative bacteria with low cytotoxicity and low susceptibility to protease K degradation (Badosa et al., 2007). According to this peptide, a novel peptide library was designed in which Lys was replaced with Arg and Trp was added as a hydrophobic residue. R-BP100 as Arg substituted analogue showed higher membrane-binding affinity over BP100. RW-BP100, besides having Arg, also contained a Trp instead of a Tyr residue. This peptide exhibited higher affinity and deeper insertion into the membrane compared to the R-BP100. These analogues were also active against gram-positive bacteria that were the consequence of deeper insertion to the membrane, resulting from the Arg and Trp residues (Torcato et al., 2013).

Conserved domains

Peptides and proteins might contain several conserved domains in their primary structures involved in construction of active structural motives. Studies showed that it would be possible to find some conserved

domains in AMPs involved in their antimicrobial, anti-inflammatory, or the other biological activities. Detection of these domains by computational methods could help scientists to design or improve the biological activities of new AMPs, such as clavainin-MO development from clavainin A and plant lipocalins. The newly designed AMP exhibited both antimicrobial and immunomodulatory features (Silva et al., 2016).

Hydrophobicity and hydrophilicity

AMPs are usually cationic due to the presence of positive charged amino acids of lysine and arginine. Besides, they compose of hydrophobic residues in about fifty percent. Therefore, they can adopt amphipathic structures, which support their interactions with and penetration to cell membranes (Zhu et al., 2015). In general, it has been recognized that increasing the peptides hydrophobicity tends to increase their toxicity (Jiang et al., 2008; Khara et al., 2015). The positions of the hydrophobic side chains and distribution of hydrophobicity and hydrophilicity are considered as important factors affecting the antimicrobial, cytotoxic, and hemolytic activities of AMPs (Liu et al., 2016). Some studies exerted amino acid mutation to alter hydrophobicity and biological activity. Murayama et al. showed that the amino acid substitution from leucine to hydrophilic glutamine at position 40 of adenovirus internal protein VI (AdVpVI, amino acids of 33–55) could change helical content but with no significant effect on their membrane perturbation ability. In addition, incorporation of phenylalanine in the hydrophobic surface exerted a significant effect on the membrane-binding, due to the shortening of helical part (Murayama et al., 2016).

Lipid chains could be considered to bind the amino acid structures and these lipo-amino acids (LAAs) have been applied in the design of novel AMP peptidomimetics. The LAA incorporated AMPs were an ultra-short peptide sequence of lysine with branches of lipid structures. This design could modulate the lipophilicity of the molecules. As a result, LAAs could combine the structural property of lipids and amino acids, and therefore, they would be easily incorporated into a peptide sequence. Some of the designed lipopeptides were highly effective against Gram-positive bacteria and MDR in addition to their low toxicity and high stability against trypsin degradation (Azmi et al., 2016).

Amino-acids modification

Natural protease in different biological systems are one of the crucial cause of AMP degradation and loss of activity. Substitution of natural L-form amino acids with unnatural D-form amino acids resulted in protease resistance of peptide drugs, such as what have been evaluated in melittin, magainin, LL-37, and several others (Chen et al.,

2016).

Substitution of D amino acids instead of L-type would result in a new feature to the AMPs activity or selectivity. Sajjan et al. demonstrated that P-113D as the mirror-image of P-113 (a histidine-rich peptides produced by the salivary glands of humans and higher primates) could be stable and active against major pathogen of the cystic fibrosis patients in the presence of sputum. This amino acid substitution could improve the peptide stability and therefore, made it a suitable drug candidate for prevention of chronic infections in patients with cystic fibrosis (Sajjan, et al., 2001). In addition, D-amino acid analogues of hexapeptide PAF19 showed stronger antifungal activities (López-García et al., 2002). One another study investigated the incorporation of D-amino acids as the unnatural amino acid to the anti-mycobacterium synthetic peptides and confirmed that this substitution was well tolerated without significant alteration in toxicity profiles and secondary conformations (Khara et al., 2016).

The other evaluated amino acid alteration is the incorporation of silicon-containing amino acids into peptides that could change the properties of the biomolecules. Accordingly, this type of substitution was applied into hydrophobic faces of alamethicin, as an antimicrobial peptide. The result confirmed that these substitutions could improve the antimicrobial activity through the enhancement of membrane permeabilization properties (Madsen et al., 2016).

Cyclization

Secondary structures of AMPs, more than amino acids composition, play an important role in their biological activity. besides α -helical structures, some of natural AMPs exhibited the β -hairpin loop, such as defensin, tachyplesin, gramicidin, polyphemusin, and protegrin, which could exert potent antimicrobial activity only if constrained by disulphide bonds. Structural analysis of these peptides had indicated the cyclic structure as the prerequisite for biological activity (Vale et al., 2014). In synthetic peptides, cyclization can enhance antimicrobial activity through improving membrane permeation (Andreev et al., 2016). Wessolowski et al. had developed RRWWRF-hexapeptide analogues by cyclization and D-amino acids substitution. In contrast to the D-substitution that had little influence on the antimicrobial activity, cyclization enhanced the activity up to four times (Wessolowski et al., 2004). The cyclization approach on the KR-12 as the short potent analogue of LL-37 confirmed the enhancement of biological activity and improvement of stability (Muhammad et al., 2016).

Branched AMPs

G3KL as an AMP dendrimer was developed at the

University of Bern (Switzerland) through sequence optimization of an initial hit compound. This branched lysine-leucine peptide sequence was identified by screening a combinatorial library of dendrimers act as a membrane-disrupting agent (Stach et al., 2012). The developed AMP dendrimer exhibited promising activity against several Gram-negative strains in addition to the low hemolytic activity and good stability in human serum (Stach et al., 2014). G3KL was also evaluated as a novel promising antibacterial agent against drug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Pires et al., 2015). In another novel approach, star-shaped peptide polymer nanoparticles had been synthesized consisting of lysine and valine residues. This structure was termed "structurally nano-engineered antimicrobial peptide polymers" or SNAPPs. The results showed that SNAPPs could be a low toxic and potent antimicrobial agent against MDR Gram-negative bacteria (Lam et al., 2016).

Future trends

The new approach for novel AMP development is the combined approach of bioinformatics and rational design. In this regard, Pearson et al. developed new AMP based on the data from both natural peptides as well as artificial peptides identified by screening of large randomly libraries. They combined *de novo* approach with rational design, and *in silico* modeling to design potent AMPs against *Mycobacterium tuberculosis* (Pearson et al., 2016). The trend in the development of novel antimicrobial peptide with improved activity and reduced toxicity is to design short cationic peptides with α -helical structure that compose several hydrophobic amino acids such as Trp. The cationic residues help the peptide to interact more specifically to bacterial membranes and the hydrophobic residues insert into the lipid bilayers of cell membrane and disturb the membrane and cell integrity. In addition, conjugation of AMPs to the nanostructures is another interesting approach in the development of new antibiotic peptide based drugs.

AMPs were also evaluated as a drug delivery vehicle for Antisense peptide nucleic acid (PNA) oligomers. Recent study had approved that AMPs with the membrane incorporation properties as the mode of action could be an efficient carrier for bacterial delivery of the PNAs targeting bacterial genes (Hansen et al., 2016). Therefore, the scientists could focus on the development of novel AMPs with selective binding to the target pathogens, which would be considered as a drug-targeting agent in addition to their therapeutic importance as an antibiotic.

Competing Interests

The authors declared that there is no conflict of interest.

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