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Review

Antimicrobial peptides: Design, chemical synthesis, activity evaluation, and application

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Abstract

Antimicrobial peptides (AMPs) are active short peptides that exist in microorganisms, insects, amphibians, plants, and mammals. Some naturally occurring AMPs have low antimicrobial activity, high haemolysis, potential toxicity toward mammalian cells, and high susceptibility to proteolytic degradation, which limit their practical application. In recent years, many efforts have been made to design and modify AMPs to improve their properties. The present review focuses on site-directed mutation, truncation, hybridisation, capping, and cyclisation of AMPs. The review further introduces the application of solid-phase peptide synthesis technology for AMPs, and summarises the methods for evaluating the antimicrobial activity of AMPs. The in-depth research on AMPs is expected to play an essential role in the fields of agriculture, animal husbandry, food industry, and medicine.

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Introduction

The constant changes in the ecological environment, and the abuse of traditional antimicrobial agents have led to the continuous evolution of microorganisms, making them more diverse and difficult to control. The emergence of drug-resistant bacteria has seriously affected the safety and health of humans and animals, and also threatened agricultural livelihoods and global food security (Kapil *et al*., 2020; Sazykin *et al*., 2021). Therefore, novel antimicrobial agents with better safety, higher efficiency, and lower drug resistance than traditional antibiotics are the need of the hour.

Antimicrobial peptides (AMPs), also known as host defence peptides (HDPs), are essential components of the innate immune system across all living organisms (Hancock *et al*., 2021). Research has shown that a large number of AMPs also exhibit antibiofilm, antiviral, antifungal, antiparasitic, anticancer, immunomodulatory, and other activities (Kang *et al*., 2019; Selvarathinam *et al*., 2021; Yu *et al*., 2021). Until now, more than 3,000 AMPs have been identified, and this number keeps increasing every year (Li *et al*., 2021a). The majority of AMPs are relatively short (< 50 amino acid residues in length), cationic, and are able to fold into amphiphilic structures when on contact with bacterial membrane (Mookherjee *et al*., 2020).

The traditional antibiotics target the receptors on the bacterial membrane or cytoplasm, which are limited in number; thus, bacteria develop drug resistance through mutations (Kapoor *et al*., 2017). The mechanism of action of AMPs does not involve specific receptors, and is quite different from that of traditional antibiotics. Most of the AMPs could act directly on the negatively charged bacterial membrane, change the permeability of the bacterial outer and inner membranes, cause physical damage to the cell membrane, and eventually lead to bacterial death (Benfield and Henriques, 2020). Some of them might exert their antimicrobial activity by inhibiting protein synthesis (Zhou and Chen, 2011), inhibiting cell wall formation (Raja *et al*., 2017), inhibiting enzyme activity (Shinnar *et al*., 2003), or interacting with DNA/RNA (Han *et al*., 2021). Although the specific mechanism of a certain AMP remains undetermined, the synergistic effect of multiple

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mechanisms is certain. AMPs could quickly kill bacteria without developing drug resistance, thus, making them an ideal alternative to conventional antibiotics.

Naturally occurring AMPs have attracted great attention for their numerous advantages; however, they still have many drawbacks to their practical application. For example, some AMPs display relatively low antimicrobial activity, high haemolysis, potential toxicity toward mammalian cells, and high susceptibility to proteolytic degradation (Schmidtchen *et al*., 2002; Bacalum and Radu, 2015; Wang *et al*., 2021). Therefore, using different technologies to design and modify AMPs and then measure their activity has become the mainstream in the development of novel AMPs. The present review emphasises on site-directed mutation, truncation, hybridisation, capping, and cyclisation of AMPs. The present review further introduces the application of solid-phase peptide synthesis technology for AMPs, and summarises the methods for evaluating the antimicrobial activity of AMPs. The application prospects of AMPs in multiple fields are also predicted.

Rational design of AMPs

In recent years, various strategies have been implemented to design novel AMPs with potent activity and low cytotoxicity (Table 1). Experimental results have shown that the rationally designed AMPs usually exhibit superior antimicrobial activity than the parental peptides without causing cytotoxicity. The common methods for designing and modifying peptides include site-directed mutation, truncation, hybridisation, capping, cyclisation, *etc*.

Site-directed mutation

Site-directed mutation of the amino acid residues, which includes substitution, movement, insertion, and deletion of one or more amino acid residues, has mediated the formation of AMPs with desired biological activities (Huan *et al*., 2020). It has been found that the basic and hydrophobic amino acids in the peptide sequence are critical for enhancing peptide activity. Peptide aurein 1.2 (GLFDIIKKIAESF-NH_2), which was isolated from the skin secretions of Australian bell frogs, exhibited antimicrobial and anticancer activities (Rozek *et al*., 2000). Migoń *et al*. (2019) investigated using alanine scanning the effects of each amino acid residue on the physicochemical and biological properties of aurein 1.2. The analogues of aurein 1.2 (containing D4A or E11A substitutions, where the negatively charged aspartic and glutamic acid residues are replaced by hydrophobic alanine) exhibited improved antimicrobial activity against both Gram-positive and Gram-negative bacteria. Ramezanzadeh *et al*. (2021) designed three aurein 1.2 analogues, namely aurein M1 (GLFDIIKKIWESF-NH_2), aurein M2 (GLFKIIKKIAKSF-NH_2), and aurein M3 (GLFKIIKKIWKSF-NH_2), by inserting hydrophobic tryptophan and cationic lysine residues into aurein 1.2. Aurein M3 showed broad-spectrum antimicrobial activity, and its antimicrobial activity was significantly higher than parental peptide aurein 1.2 and the other two analogues. These results suggested that the introduction of favourable amino acids into the peptide sequence contributed to enhancing the antimicrobial activity of the AMPs. The AMP Hp1404, identified from the venom of the scorpion *Heterometrus petersii*, displayed certain cytotoxicity toward mammalian cells (Li *et al*., 2014). Kim *et al*. (2018) designed a set of Hp1404 analogues to reduce its cytotoxicity by replacing glycine and phenylalanine with leucine and lysine residues. The resulting analogues showed less haemolysis and toxicity toward mammalian cells when compared with peptide Hp1404.

Truncation

The cost of synthetic AMPs is relatively high due to a large number of amino acid residues and complex structures. Truncating the heavy chain of the parental peptides, as well as retaining their active fragment, is a promising strategy for AMP optimisation. Dong *et al*. (2012) truncated the C-terminus of linear avian β-defensin-4 to obtain peptide GL23. The cysteine in GL23 was then replaced with isoleucine to obtain a novel derivative peptide GLI23. The peptide GLI23 exhibited excellent antimicrobial activity and relatively low haemolysis when compared with avian β-defensin-4. Salasa *et al*. (2018) designed five peptides (pal-ano-9 to pal-ano-5) by truncating the C-terminus of palmitoylated anoplin (pal-anoplin). The analogues, namely pal-ano-9 and pal-ano-6, significantly improved their activity against the fungus *C. albicans*. In addition to retaining or improving the antimicrobial activity, the truncated AMPs could sometimes reduce their toxicity toward mammalian

Table 1. Antimicrobial activities of the rationally designed AMPs against common pathogens.

Peptide analogue	Parental peptide	Pathogen	MICs of analogue (or parental peptide)	Reference
GLI23	β-defensin-4 (RL38)	*E. coli*, *S. pullorum*, *S.* Typhimurium, *S. aureus*, *S.* Epidermidis, *S. faecalis*	2 - 8[b] (2 - 8[b])	Dong *et al.* (2012)
Syn-GNU7, Lf-GNU7, and BPI-GNU7	GNU7	*M. luteus*, *S. aureus*, *E. coli*, *S.* Typhimurium	0.5 - 4[b] (1 - 32[b])	Kim *et al.* (2016)
Cecropin A(1-8)-LL37(17-30)	Cecropin A, LL-37	*S. aureus*, *E. coli*, *L. monocytogenes*, *M. luteus*	2 - 18[a] (4 - 198[a])	Wei *et al.* (2016)
pal-ano-9, and pal-ano-6	Pal-anoplin	*E. coli*, *S. aureus*, *C. albicans*	0.406 - 3.25[a] (3.25 - 6.5[a])	Salasa *et al.* (2018)
C12O3TR	O3TR	*F. culmorum*, *A. niger*, *P. expansum*, *Z. bailii*, *Z. rouxii*, *K. lactis*, *D. hansenii*, *S. cerevisiae*	3.12 - 25[a] (12.5 - 50[a])	Thery *et al.* (2018)
cTI, cTII, and cTIII	Tachyplesin TI, TII, TIII	*E. coli*, *S. aureus*	1 - 8[b] (0.0625 - 8[b])	Vernen *et al.* (2019)
R7I	*De novo* design	*E. coli*, *S. pullorum*, *S.* Typhimurium, *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*	1 - 64[b]	Wang *et al.* (2019)
C10-D4,7, and C12-D4,7	Anoplin	*E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* MRSA, *B. subtilis*	2 - 16[b] (8 - 64[b])	Zhong *et al.* (2019)
RI12[K3W]	RI12	*E. coli*, *S.* Typhimurium, *S. pullorum*, *S. aureus*, *S. epidermidis*, *E. faecalis*, *B. subtilis*	2 - 128[b] (1 - 4[b])	Lyu *et al.* (2020)
Pep	*De novo* design	*E. coli*, *P. aeruginosa*, *Pseudomonas* sp., *S. aureus*, *Klebsiella*, *B. subtilis*, *Acinetobacter* sp., *Citrobacter* sp.	(2 - 4[b])	Thankappan *et al.* (2021)
C4-CAMEL, C8-CAMEL, and C12-CAMEL	CAMEL	*E. coli*, *S. aureus*	4 - 32[b] (2 - 8[b])	Ma *et al.* (2022)
CMt1, and CMt2	Cecropin, A-Magenin 2	*E. coli*, *P. aeruginosa*, *C. krusei*, *P. pinophilum*, *T. mentagrophytes*, *S. cerevisiae*	7.81 - 62.5[a] (7.81 - 125[a])	Namvar Arabani *et al.* (2022)

[a]MIC: minimum inhibitory concentration in µg/mL; [b] MIC: minimum inhibitory concentration in µM.

cells. Peptide P5 (KWKKLLKKPLLKKLLKKL-NH_2), a cecropin A-magainin 2 hybrid peptide, showed high antimicrobial activity against Gram-positive / Gram-negative bacteria (Park *et al*., 2003). Six truncated peptides, namely P5-CT1, P5-CT2, P5-CT3, P5-NT1, P5-NT2, and P5-NT3, were obtained by truncating two, four, and six amino acid residues at the C- or N-terminus of the peptide P5, respectively. These truncated peptides exhibited low haemolysis and cytotoxicity when compared with the parental peptide P5 (Kwon *et al*., 2019). Lin *et al*. (2013) obtained a series of truncated peptides by truncating peptide shrimp anti-lipopolysaccharide factor (SALF), epinecidin (Ep), and pardaxin (GE). The resulting peptides Ep-8 and GE-6 had a broad-spectrum antimicrobial activity. Also, the combination of the truncated AMPs and non-peptide antibiotics reduced the minimum inhibitory concentrations (MICs) of peptides Ep-1, GE-1, GE-6, and Ep-8 against methicillin-resistant *S. aureus* (MRSA). Peptide LL-37 exhibited spermicidal activity against human and mouse sperm, as well as microbicidal activity against various sexually transmitted infection (STI) pathogens (Srakaew *et al*., 2014; Tanphaichitr *et al*., 2016); however, LL-37 contains 37 amino acid residues, which greatly increased the difficulty of its synthesis. Two truncated peptides, namely GI-20 and GF-17, located in the central helical structure of LL-37, exhibited spermicidal and microbicidal activity similar to LL-37 (Kiattiburut *et al*., 2018). Lin *et al*. (2022) compared the antimicrobial activity of the truncated peptides pardaxin (1-22), MSI-78 (4-20), DMPC (1-19), and cecropin B (1-21), and explored their antimicrobial mechanisms, and found that these peptides primarily exerted their activity through membrane destabilisation and disruption.

Hybridisation

Fink *et al*. (1989) proposed the theory of hybrid AMPs based on the antimicrobial mechanism of cecropin A and the effect of amino acid substitution on antimicrobial activity. Hybrid AMPs were reconstructed by covalently linking two or more peptide fragments, in which the advantages of each fragment were integrated, and the biological activity of AMPs was improved. Cecropin A, a well-studied AMPs, had strong antimicrobial activity, low toxicity, and high stability. The N-terminal α-helix domain of cecropin A has been widely used to hybrid with the core sequence of other AMPs (*e.g*., LL37, magainin, melittin) to develop novel hybrid AMPs. Boman *et al*. (1989) designed a series of cecropin A-melittin hybrid peptides, namely CA(1-24)-M(1-13), CA(1-13)-M(1-13), CA(25-37)-CA(1-13), M(1-13)-CA(1-13), and CA(1-8)-M(1-18), where the haemolysis of these hybrid peptides was significantly lower than the parental peptide melittin, and the antimicrobial activity was similar to cecropin A and melittin. The hybrid peptide cecropin A(1-8)-LL37(17-30) was obtained by connecting residues 1 to 8 of cecropin A, with residues 17 to 30 of peptide LL37, and exhibited improved antimicrobial activity against indicator strains and lower haemolysis in sheep erythrocytes (Wei *et al*., 2016). Hybrid peptide magainin-thanatin (MT) (Tian *et al*., 2019) was constructed by combining residues 1 to 12 of magainin with residues 5 to 21 of thanatin. The MICs of hybrid peptide MT against *S. aureus*, *E. coli* DH5-α, and *B. subtilis* were 16.5, 20, and 9 μM, respectively, which were lower than the parental peptides. The theory of hybrid AMPs can further be used to design specifically targeted antimicrobial peptides (STAMPs), where the peptides with targeting function are hybridised with broad-spectrum AMPs. STAMPs identified and killed the target bacteria to achieve the purpose of targeted therapy. Kim *et al*. (2016) designed three hybrid peptides by connecting LPS-targeting peptides lactoferrin (Lf 28-34), BPI 84-99, or *de novo* peptide (Syn) with the AMP GNU7 using a flexible linker. The resulting hybrid peptides Lf-GUN7, BPI-GNU7, and Syn-GNU7 displayed 8- to 32-fold improvement in antimicrobial activity against Gram-negative bacteria than the parental peptide GNU7. Syn-GNU7 showed the strongest LPS-binding and LPS-neutralising activities, and selectively eliminated Gram-negative bacteria from the mixed culture.

Capping

The AMPs with N- or C-terminal bare are easily recognised and cleaved by proteases, resulting in degradation or loss of activity. Therefore, the N- or C-terminal capping improved AMP stability and activity. The hydrophobic end modification of AMPs, a general capping method, is achieved by attaching an acyl group or a hydrophobic amino acid stretch to the N- or C-terminus of the parental peptides. The capping of AMPs also promoted the AMPs to bind or insert themselves into the lipid membrane, thus causing bacterial death (Schmidtchen *et al*., 2014). The addition of a variable length fatty acid tail to the

N-terminus of AMPs has been regarded as an effective method to modulate peptide activity and selectivity. Zhong *et al*. (2019) designed and synthesised a series of anoplin analogues by conjugating fatty acids to the N-terminus of partial D-amino acid substitution analogues of anoplin and its dimer. The analogues exhibited pronounced antimicrobial activity against the tested Gram-positive and Gram-negative bacteria. Nielsen *et al*. (2022) designed a series of peptoids with lipid tails or halogen substituents. The small-angle X-ray scattering showed that these peptoids self-assembled into different nanostructures. The authors then explored the relationship between self-assembly structures and biological activity, and proved that certain self-assembled morphologies contributed to improving antimicrobial activity. The end-tagging with hydrophobic amino acid stretches (especially tryptophan and phenylalanine) was another interesting approach to improve peptide antimicrobial activity. The C-terminus of peptide GRRPRPRPRP was tagged with four aromatic amino acids, and the obtained peptide GRR10W4NH2 (GRRPRPRPRPWWWW-NH_2) displayed a broad-spectrum antimicrobial activity and low toxicity toward human cells (Schmidtchen *et al*., 2011).

Cyclisation

Cyclopeptides, as compared to linear peptides, had a higher affinity for receptor subtypes, and were more resistant to proteases due to the constrained conformation of the backbone of cyclopeptide molecules. The lipopeptides colistin and daptomycin were found to be effective in the treatment of multidrug-resistant Gram-negative and Gram-positive bacteria, and both contain a cyclic peptide part that is critical for their activity (Falagas and Kasiakou, 2005; Humphries *et al*., 2013). Oh *et al*. (2014) reported two classes of amphiphilic cyclic cell-penetrating peptides (CPPs), which showed potent antimicrobial activity. The cyclic peptide [R4W4] specifically displayed the strongest antimicrobial activity against methicillin-resistant *S. aureus*, with a MIC of 2.67 μg/mL, while exerting the expected cell-penetrating properties toward eukaryotic cells. Vernen *et al*. (2019) designed three cyclic analogues cTI, cTII, and cTIII based on the backbone cyclisation of tachyplesin I, II, and III, respectively. Although these three cyclic analogues had similar structure and activity against bacteria and cancerous cells with linear tachyplesin; the cyclic analogues had reduced toxicity toward human red blood cells, and also increased their stability in human serum. Thomsen *et al*. (2020) designed and synthesised 18 analogues of cyclic peptide BSI-9 based on the differences in cyclisation point, hydrophobicity, and cationic side-chain length. Analogue 11 showed improved inhibitory activity against *S. aureus* and *P. aeruginosa*, with MICs of 8 and 4 μg/mL, respectively. Cyclic amphiphilic peptides [W4KR5] obtained by N- to C-cyclisation of linear peptide W4KR5 had improved activity as compared to linear peptide W4KR5. The combination of [W4KR5] with other antibiotics significantly improved the antimicrobial effect, and could be used to treat multidrug-resistant pathogens (Mohammed *et al*., 2022).

Others

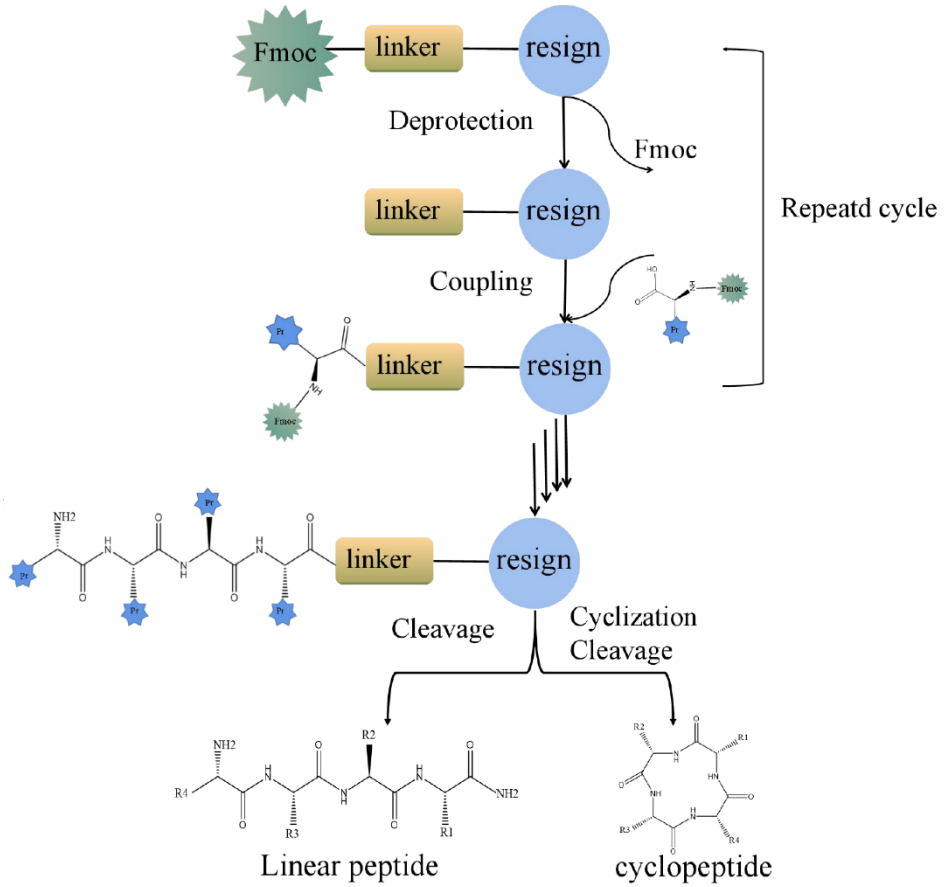
Some of the other effective methods to design and modify AMPs include the introduction of unnatural amino acids, PEGylation, *de novo* design, computer design, halogenation, and others. Oliva *et al*. (2018) designed three cationic peptides containing unnatural amino acids, which displayed high stability in serum and had broad-spectrum antimicrobial activity. Wang *et al*. (2019) used a minimalist *de novo* design approach to establish an antitrypsin / antichymotrypsin hydrolytic peptide sequence unit, namely $(XYPX)_n$ (X = isoleucine, leucine, or valine; Y = lysine, or arginine; and n is the number of repeat units). Among these designed peptides, peptide 16 with $(IRPI)_7$ sequence had the highest average selectivity index (GM_{SI}) against all of the tested Gram-negative bacteria, with a GM_{SI} of 99.07. Importantly, peptide 16 also had resistance to trypsin / chymotrypsin hydrolysis at higher concentrations. Nagarajan *et al*. (2018) designed a series of AMPs based on a long short-term memory (LSTM). All of these designed peptides displayed broad-spectrum antimicrobial activity. Kaur *et al*. (2020) prepared peptide cryptdin-2 in *E. coli* using recombinant DNA technology, and then a PEG-conjugated variant of cryptdin-2 was obtained using thiol-specific PEGylation. The obtained PEGylated cryptdin-2 had good serum stability and low toxicity, and could be used in combination with other conventional antibiotics.

Chemical synthesis of AMPs

Direct extraction (Mandal *et al*., 2009), enzymatic hydrolysis (Real Hernandez and Gonzalez

De Mejia, 2019), recombinant DNA technology (Li, 2011; Zhan *et al*., 2020), and other methods (He *et al*., 2016; Przybylski *et al*., 2020) could be used to acquire AMPs. However, most of these methods were complex, time-consuming, and low yield, which make them suitable only for laboratory research than large-scale production. Importantly, some precisely modified AMPs (*e.g*., unnatural amino acid, fatty acid, aromatic acid, PEG, *etc*.) were difficult to achieve using the above methods. Therefore, chemical synthesis gradually became the mainstream of peptide preparation technology, which could be used to synthesise AMPs with high yield, complex structure, and accurate modification. Li *et al*. (2021b) nicely reviewed the chemical modifications of AMPs, *e.g*., lipidation, glycosylation, N-/C-terminal unusual modifications, and conventional antibiotics conjugation; and the authors also mentioned the examples of solid-phase synthesis for these AMPs.

Merrifield (1963) proposed the solid-phase peptide synthesis (SPPS) technology developed on the basis of liquid-phase peptide synthesis (LPPS). SPPS, carried out on a solid support, included the process of repeatedly adding amino acids from the C-terminus (carboxyl terminus) to the N-terminus (amino terminus) (Scheme 1). To prevent the occurrence of side reactions, the side chains of the amino acids were protected. When compared with LPPS, SPPS had significant advantages of being rapid, efficient, simple, high yield, automatic, *etc*. (Amblard *et al*., 2006). Boc and Fmoc strategy were the two main strategies for SPPS. In general, SPPS could efficiently synthesise both linear and cyclic peptides.



Scheme 1. Schematic illustration of solid-phase peptide synthesis (SPPS). Take amide-MBHA resin as an example, Fmoc: amino protecting groups; Pr: side chain protecting groups.

Solid-phase synthesis of linear peptide

SPPS is a relatively mature method for synthesising linear peptides. Linear peptide Cn-AMP1 isolated from green coconut water (*Cocos nucifera*) exerted activity against *E. coli*, *B. subtilis*, *S. aureus*, and *P. aeruginosa* (Mandal *et al*., 2009). Cn-AMP1 was synthesised by SPPS using the Fmoc strategy, and showed a wide range of activities, including antimicrobial and immunostimulatory (Silva *et al*., 2012). Anaya *et al*. (2020) revealed that the synthetic Cn-AMP1 showed little cytotoxicity toward LS180 and Caco-2 cell lines, and had no effect on the expression and activity of P-glycoprotein. Zhao *et al*. (2021) synthesised a series of BRCA1

(856-871) analogues containing 16 amino acid residues using the SPPS method, which were characterised by reversed-phase high-performance liquid chromatography (RP-HPLC) and electron spray ionisation mass spectrometry (ESI-MS). SPPS had been widely used to synthesise non-amino acid-modified linear peptides (*e.g*., PEGylation, lipidation, acetylation, phosphorylation, glycosylation, *etc*.). Ardila-Chantré *et al*. (2020) synthesised a group of short peptides modified with non-peptide molecules (6-aminohexanoic acid, ferrocene, caffeic acid, ferulic acid, and oxolinic acid) by SPPS using the Fmoc strategy. The synthetic peptide conjugates were purified by reversed-phase solid-phase extraction (RP-SPE), and characterised by RP-HPLC, MALDI-TOF MS, and circular dichroism spectrum. These conjugates exhibited higher antimicrobial activity than the original unconjugated peptides. Paquet-Côté *et al*. (2020) synthesised three simplified model peptides that bore crown ethers. These peptides were characterised by HPLC and high-resolution mass spectra (HR-MS). The effects of the peptide length and crown ether ring size on their secondary structures and activity were studied. Nielsen *et al*. (2022) synthesised a set of peptoids with lipid tails or halogen substituents using a sub-monomer approach by sub-monomer solid-phase synthesis with a cocktail of trifluoroacetic acid, triisopropylsilane, and water being used as a cleavage reagent. The synthetic peptoids were purified using preparative HPLC, and characterised by analytical UPLC/MS. The small-angle X-ray scattering results showed that these peptoids self-assembled to different morphologies. Peptoids capable of forming ellipsoids or bundled assemblies exhibited improved antibacterial, antibiofilm, and anti-abscess activities.

Solid-phase synthesis of cyclopeptides

Cyclopeptides synthesised by solid-phase synthesis could avoid inter-molecular dimerisation or multimerisation. As per the cyclisation method, it can be divided into head-to-tail, sidechain-to-sidechain, sidechain-to-end, and disulphide-bridge. Tamaki *et al*. (2006) synthesised the precursor of GS (H-D-Phe-Pro-Val-Orn-Leu-oxime) on a resin (loading of oxime group: 0.48 mmol/g) by SPPS using the Boc strategy, and then cyclised it in different solutions. The experimental results showed that from among these solvents, the cyclisation in 1,4-dioxane gave the best yield. Thomsen *et al*. (2020) synthesised 18 cyclic peptide analogues based on the peptide BSI-9 on a TentaGel®S RAM (90 μm) resin with a loading of 0.22 mmol/g. The cleavage was performed with a TFA:H_2O:TIS (95:2.5:2.5, v/v) solution for 2 h. Peptide 11 (Dab3 -> Arg) exhibited improved activity against *S. aureus* and *P. aeruginosa*, with MICs of 4 and 8 μg/mL, respectively. Qu *et al*. (2020) used the native chemical ligation (NCL)-assisted diaminodiacid (DADA) strategy to synthesise disulphide surrogate peptides. The peptide sequences were firstly synthesised using Fmoc SPPS and the intramolecular cyclisation using hydraside-based NCL. The stability studies indicated that the disulphide replacements could overcome disulphide reduction and scrambling. Bicyclic peptides, with dual conformational constraints and rigid structures, are more resistant to proteolytic degradation as compared to linear and monocyclic peptides (Ahangarzadeh *et al*., 2019). Jaradat *et al.* (2019) synthesised a cyclopeptide containing two disulphide bonds on a resin by orthogonal protection approach, and the cyclopeptide was analysed and characterised by LC-MS/MS and HPLC. The purified peptide showed excellent antimicrobial activity against the tested Gram-positive and Gram-negative bacteria. Zhu *et al*. (2021a) prepared a thioether-bonded bicyclic peptide using Trt- and StBu-protected cysteines. Chen *et al*. (2021) synthesised a linear peptide by SPPS, and then cross-linked the peptide head-to-tail and sidechain-to-sidechain to obtain a group of bicyclic helical peptides. For the head-to-tail cross-linking, the N-terminal amino group and the C-terminal carboxyl group were modified with 3-thiopropionic acid (MPA) and 2-mercaptoethylamine (MEA), respectively, and then were connected by 1,4-dibromomethylbenzene. For the sidechain-to-sidechain, the amino acid residues were mutated to cysteine at positions i and i+3, or positions i and i+4, and were cross-linked by 1,3-dibromomethylbenzene. These bicyclic peptides had enhanced proteolytic stability, helicity, and bioactivity.

Antimicrobial activity evaluation

The antimicrobial activity of AMPs could be evaluated by multiple methods; however, existing studies on AMPs focused on *in vitro* and *in vivo* antimicrobial assays (Fuscaldi *et al*., 2021).

In vitro antimicrobial activity evaluation

In vitro antimicrobial experiments have a short duration, good repeatability, rapidity, and

convenience. It can be used to get the antimicrobial spectrum of the AMPs, and screen out the highly active AMPs in a short time (Elyass *et al*., 2021). However, *in vitro* antimicrobial activity lacks a unified standard method due to a large number of AMPs and their different physicochemical properties. The most common methods for evaluating the *in vitro* antimicrobial activity are the agar diffusion method (Fisher *et al*., 2016) and broth microdilution method (Otvos and Cudic, 2007).

The activity of peptide Brevinin-2GUb and its analogues against Gram-positive bacteria (*e.g*., *S. aureus* MRSA, *E. faecalis*), Gram-negative bacteria (*E. coli*, *K. pneumoniae*, *P. aeruginosa*), yeast (*C. albicans*), and clinical isolates MRSA were evaluated by the broth microdilution method (Lin *et al*., 2021). A series of two-fold peptide dilutions were added to a 96-well plate, and incubated with bacterial cells at 37°C overnight. The minimum inhibitory concentrations (MICs) of the peptides were determined from the optical density using a microplate reader. The minimum bactericidal concentrations (MBCs) were obtained by spreading 10 μL of each clear well onto a Mueller Hinton Agar (MHA) plate. The MICs and MBCs of Brevinin-2GUb and its analogues against those microorganisms helped the authors to screen out a peptide analogue, tB2U-6K, which had higher activity than Brevinin-2GUb and other analogues. Fang *et al*. (2019) qualitatively and quantitatively compared the activity of octopus scraps peptides-zinc chelate (OSPH-Zn) and zinc salts against *S. aureus* by agar disk diffusion method and broth microdilution method. The sterile filter paper discs containing OSPH-Zn (100 mg/mL) or zinc salts (100 mg/mL) were placed on the Luria-Bertani Agar (LBA) plate, and inoculated with bacterial suspension at 37°C for 18 h. In the presence of OSPH-Zn and zinc salts, the inhibition zone areas were 482.93 ± 22.03 and 289.51 ± 17.05 mm^2, respectively. These results showed that OSPH-Zn had higher antimicrobial activity than inorganic zinc salts against *S. aureus*. The MIC of OSPH-Zn against *S. aureus* was 1.56 mg/mL, which was significantly lower than that of inorganic zinc salts, thus confirming the results of the agar disk diffusion method. Wang *et al*. (2020b) investigated the susceptibilities of bacteria to peptide $CAMP_{211-225}$ using *E. coli*, *Y. enterocolitica*, *L. monocytogenes*, *K. pneumoniae*, *S. aureus*, and *B. subtilis* as the indicator bacteria. The broth microdilution method was used to determine the MICs of $CAMP_{211-225}$ against the six pathogenic bacteria. The results showed that the MICs of $CAMP_{211-225}$ against *E. coli* and *Y. enterocolitica* were 3.125 and 6.25 μg/mL, respectively. However, $CAMP_{211-225}$ failed to show activity against the other three bacteria at a concentration of 50 μg/mL. Furthermore, the activity of $CAMP_{211-225}$ against *E. coli* and *Y. enterocolitica* was confirmed by disk diffusion assay. The diameters of the inhibition zone were 7 - 16 mm (mean 10.43 mm) for *E. coli* and 8 - 15 mm (mean 10.56 mm) for *Y. enterocolitica*. The $CAMP_{211-225}$ thus presented potent activity against pathogenic *E. coli* and *Y. enterocolitica*, which was consistent with the conclusion obtained by the broth microdilution method.

In vivo antimicrobial activity evaluation

In vivo, antimicrobial experiments using animal models, further validated the antimicrobial activity of the AMPs under physiological conditions, which is more convincing than *in vitro* experiments. *In vivo* antimicrobial experiments prepared an important foundation for the clinical application of AMPs. At present, the animal models used for the *in vivo* antimicrobial studies are mainly murine, including ICR mouse, Wistar rat, Holtzman rat, CD-1 mouse, CFW-1 mouse, *etc*. (Cirioni *et al*., 2008; Wu *et al*., 2021).

Zhu *et al*. (2019) investigated the activity of the combination of AMPs HPRP-A1, HPRP-A2, and chlorhexidine acetate (CHA) against bacteria and the fungus *C. albicans* in mouse and rat vaginitis infection models. The adult female ICR mice (20 - 25 g) and Wistar rats (180 - 200 g) were used to construct the models of mouse *C. albicans* vaginitis infection and rat bacterial (*E. coli* and *S. aureus*) vaginitis infection, respectively. The efficacy was assessed by bacterial count, external vaginal surface appearance, and allergic reactions. The results showed that the combination of AMPs HPRP-A1, HPRP-A2, and CHA had strong synergistic effects on mouse and rat vaginitis infection models. The combination of HPRP-A2 and CHA, specifically, could achieve 99.9% of inhibition against infections in gynaecological vaginitis caused by bacteria and fungus in both the rat and mouse models. Wu *et al*. (2021) selected healthy ICR mice aged 4 - 5 weeks to establish a sepsis model by intraperitoneal injection of amoxicillin-resistant *E. coli* 7,000,853,626. The mice were intraperitoneally injected with peptide PCL-1 twice at 0.5 and 2 h after infection. After

infection for 8 h, the bacterial burden in the tissues and blood was tested by survival rate and bacterial titres. The results showed that PCL-1 effectively improved the survival rate of mice that were systemically infected with drug-resistant *E. coli* by effectively removing bacteria from blood and organs.

Application potential of AMPs

AMPs generally have broad-spectrum antimicrobial properties, high stability, and low cytotoxicity. Therefore, AMPs have potential application prospects in the fields of agriculture, animal husbandry, food industry, and medicine (Figure 1).

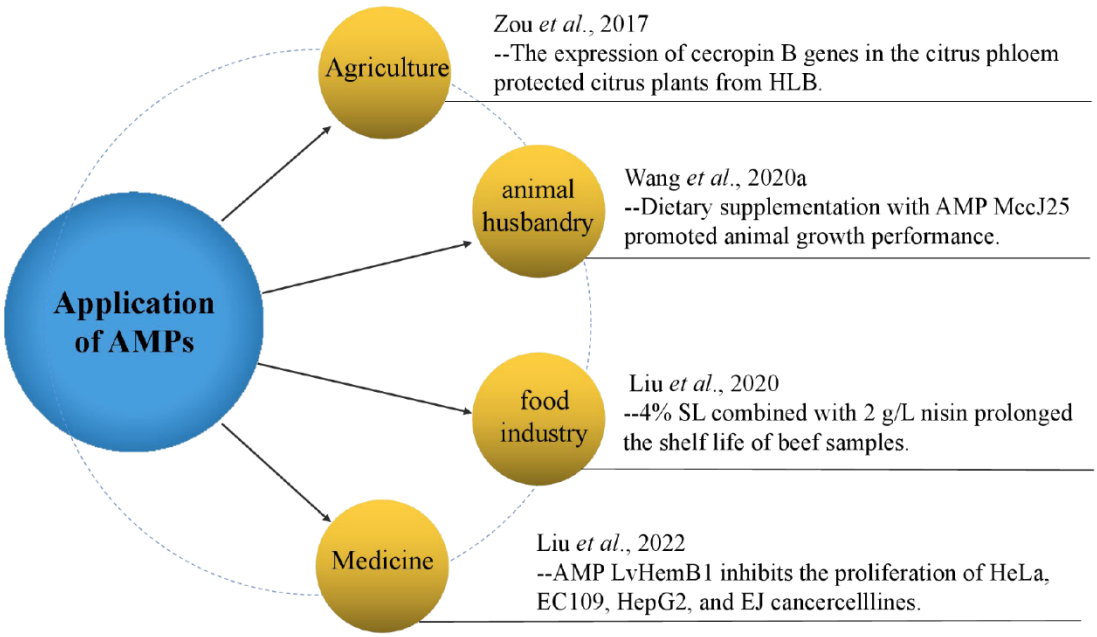


Figure 1. Applications of AMPs in multiple fields.

Applications in agriculture

Pest control has been an important measure for reducing crop damage and increasing crop yield. Transgenic crops resist different kinds of pathogens, and effectively reduce the use of chemical pesticides (Panopoulos *et al*., 1996). Researchers have been trying to transfer antimicrobial peptide genes into crops to develop new disease-resistant varieties, which have great significance in reducing agricultural production costs and protecting the ecological environment.

Radish defensin Rs-AFP2 was efficiently expressed in tobacco and tomato, and the obtained transgenic plants could resist *Alternaria longipes* (Terras *et al*., 1995). The expression of the cecropin B gene in the phloem tissues of transgenic plants decreased the host's susceptibility to Huanglongbing (HLB) (Zou *et al*., 2017). The antimicrobial peptide genes *Shiva* A and *Cecropin* B were introduced into the genome of sweet orange by *Agrobacterium tumefaciens*-mediated transformation and regeneration of mature axillary buds. Artificial inoculation experiments further indicated that the resistance of transgenic plants to *X. axonopodis* pv. *citri* was enhanced in comparison with non-transgenic lines (He *et al*., 2011). Biliarski *et al*. (2020) expressed two fusion lytic peptides, ORF13 and RSL1, in *Nicotiana benthamiana* tobacco plants, and then tested their resistance activity against three fungal pathogens, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, and *Pythium* species. The symptom area of each leaf was measured, and found that the transgenic plant lines ORF13 and RSL1 had resistance against *Sclerotinia sclerotiorum* and *Rhizoctonia solani*.

Applications in animal husbandry

Antibiotics have played an important role in preventing and treating diseases and ensuring the development of animal husbandry. However, long-term abuse of antibiotics in animal husbandry has resulted in many serious problems, *e.g*., bacterial drug resistance, antibiotic residues, and ecological pollution (Bacanli and Başaran, 2019). The European Union banned the use of antibiotic growth promoters in animal feed in 2006. Later, many other countries

also introduced policies to limit the use of antibiotics. Therefore, seeking green and pollution-free alternatives to antibiotics became an inevitable choice.

AMP Microcin J25 (MccJ25), produced by a faecal strain of *E. coli*, demonstrated strong activity against *E. coli* (Sable *et al*., 2000). Dietary supplementation with MccJ25 effectively promoted animal growth performance, enhanced intestinal barrier function, improved faecal microbiota composition, and attenuated diarrhoea and systematic inflammation (Yu *et al*., 2017; Wang *et al*., 2020a). Shi *et al*. (2018) added composite antimicrobial peptide (CAP) into the basal diet of weaned piglets. The results showed that CAP maintained intestinal microflora homeostasis, ameliorated the faecal microflora, increased the apparent digestibility of nutrients, and enhanced the growth performance and health status of weaned piglets. Bovine respiratory pathogen, *Histophilus somni*, is one of the most important bacterial pathogens associated with the bovine respiratory disease complex (BRDC). Dassanayake *et al*. (2017) synthesised a series of bovine NK-lysin-derived peptides, namely NK1, NK2A, NK2B, and NK2C, which were effective in killing *Histophilus somni* at 10 - 30 μM. The mixed formulations of AMPs and other additives were effective in improving the growth performance and immune protection of poultry (Wang *et al*., 2011). The mixture of *Laminaria japonica* powder (LJP) and cecropin used as feed supplements significantly inhibited the growth of *E. coli*, and promoted the growth of *Lactobacillus*, thereby improving the growth performance and immune function of broilers (Bai *et al*., 2019).

Applications in food industry

Food preservatives are critical for food safety, which can prevent food spoilage and prolong the food shelf life. However, in recent years, some reports have shown that long-term excessive use of traditional chemical preservatives (*e.g*., nitrite and sulphur dioxide) caused adverse effects on human health. Therefore, the development of natural preservatives became important for the food industry. Recently, AMPs nisin (Shin *et al*., 2016) and ε-poly-L-lysine (Chheda and Vernekar, 2015) have been approved as food preservatives in some countries, and certified as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration (FDA).

Nisin has been widely used in various foods, such as milk, dairy desserts, processed cheeses, meats, canned foods, alcoholic beverages, *etc*. (de Souza de Azevedo *et al*., 2019). The antimicrobial activity of nisin is, however, affected by unfavourable factors, *e.g*., an alkaline environment, high temperature, and some specific protease. Therefore, great efforts are devoted to improving the stability of the antimicrobial activity of nisin. The antimicrobial activity and stability of the covalently bonded gellan gum with nisin improved against *S. epidermidis* and *B. subtilis* when compared with nisin alone (Peng *et al*., 2020). Nisin nanoparticles, prepared using a facile nanoprecipitation technique, exhibited higher antimicrobial activity than free nisin after autoclaving at 121°C for 20 min (Chang *et al*., 2018). A single food preservative usually exerted its antimicrobial effect on a specific spoilage bacterium, and had no or weak inhibitory effect on other bacteria. When different types of preservatives were used in combination, the synergistic effect enhanced their antimicrobial effect, reduced the amount of a single preservative used, and reduced the production cost. Previous reports had shown that chitosan extracted from shrimp shells exhibited excellent antimicrobial activity, and prolonged the food shelf life (Abdel-Rahman *et al*., 2015; Dotto *et al*., 2015). The combination of 1% chitosan and 0.6% nisin demonstrated quality improvement of the large yellow croaker, *e.g*., moisture loss control, volatile spoilage inhibition, TVB-N reduction, TVC growth control, colour, and sensory acceptability maintenance (Hui *et al*., 2016). Peng *et al*. (2018) used 1% chitosan and different concentrations of nisin as preservatives to investigate their effect on the quality of jumbo squid (*Dosidicus gigas*) during cold storage. The results showed that 1% chitosan combined with 6 g/L nisin effectively inhibited the reproduction of microorganisms, and the degradation of nutrients. Meanwhile, it maintained high sensory scores, low moisture loss, and volatile spoilage products. Liu *et al*. (2020) evaluated the effect of sodium lactate (SL) coating enriched with nisin on beef, where 4% SL combined with 2 g/L nisin prolonged the shelf life of beef samples.

The peptides r(P)ApoB$_L^{Pro}$, r(P)ApoB$_L^{Ala}$, and r(P)ApoB$_S^{Pro}$ derived from human apolipoprotein B-100 (ApoB), exerted a wide range of properties, *e.g*., antimicrobial, antibiofilm, wound healing, and immunomodulatory (Gaglione *et al*., 2020).

Dell'Olmo *et al*. (2021) prepared the coating solutions composed of chitosan and ApoB-derived peptides. The coating solutions prevented *Salmonella* cells from adhering to various common surfaces in food manufacturing environment, and inhibited the proliferation of microorganisms in chicken meat samples. AMP C12O3TR, obtained by adding lauric acid to the N-terminus of peptide H-Orn-Orn-Trp-Trp-NH2 (O3TR), exhibited 2- to 8-fold more activity than O3TR against filamentous fungi (*F. culmorum*, *P. expansum*, and *A. niger*) and yeast (*S. cerevisiae*, *Z. bailii*, *Z. rouxii*, *D. hansenii*, and *K. lactis*). After seven days, C12O3TR effectively inhibited the growth of yeast and *A. niger* in commercial lager, carbonated soft drink, and apple juice, while O3TR inhibited the yeast in commercial lager and carbonated soft drink (Thery *et al*., 2018). Nie *et al*. (2021) designed four novel chimeric lysins (P361, P362, P371, and P372) by fusing *Salmonella* phage lysin with AMP LeuA-P. The combination of P362, P372, and potassium sorbate effectively reduced the microbial counts in contaminated chilled chicken and extended the shelf life by seven days.

Applications in medicine

AMPs have become important candidates for new antimicrobial, antiviral, and anticancer drugs due to their excellent physicochemical properties and biological activities. Moreover, AMPs also have the properties of immunomodulating, neutralising endotoxin, and promoting cell division. The U.S. Food and Drug Administration (FDA) has approved several AMPs, including colistin, gramicidin D, daptomycin, polymyxin B, vancomycin, and oritavancin as clinical infection therapeutics.

The human peptide LL-37 produced by neutrophils and epithelial cells had an inhibitory effect on Gram-positive and Gram-negative bacteria (Nilsson, 2020), and also played an important role in wound healing (Ramos *et al*., 2011), angiogenesis, and arteriogenesis (Koczulla *et al*., 2003). Peptide LvHemB1 induced apoptosis by permeating cells and targeting mitochondrial voltage-dependent anion channel 1 (VDAC1); thus, it could be used as an anticancer agent for the treatment of human cervical (HeLa), oesophageal (EC109), hepatocellular (HepG2), and bladder (EJ) cancer cell lines (Liu *et al*., 2022). Specifically targeted antimicrobial peptides (STAMPs) promoted AMPs to specifically target tumour tissue and cells, and achieved the goals of targeted therapy and killing. Wang *et al*. (2020c) connected the LPS-targeting peptide LBP14 with the killing domain N6 *via* different linkers to generate LPS-targeted chimeric peptides (SCPs)-A6 and G6. The SCPs-A6 and G6 improved the mouse survival rate, and alleviated lung injuries by blocking mitogen-activated protein kinase and nuclear factor kappa-B p65 activation. Moreover, SCPs-A6 and G6 showed remarkable efficacy as antimicrobial and anti-endotoxin agents in the treatment of bacterial infection and sepsis. Drug combination treatment was one of the most effective methods against microorganisms, viruses, and cancers in clinical setting (Zerweck *et al*., 2017). The combination of AMPs HPRP-A1, HPRP-A2, and chlorhexidine acetate (CHA) showed synergistic effects against bacteria, fungus, and biofilms *in vitro* and *in vivo*. In particular, the combination of HPRP-A2 and CHA had a significant inhibitory effect on the gynaecological vaginitis infection caused by bacteria, *C. albicans*, or biofilms in rat and mouse vaginitis models (Zhu *et al*., 2019; 2021b).

Conclusion

AMPs have become an attractive candidate for the development of novel antimicrobial agents due to their numerous advantages. In the present review, we demonstrated a number of effective strategies for designing and modifying AMPs to enhance their performance. Additionally, we highlighted the application of solid-phase peptide synthesis technology in peptide preparation, and summarised the methods for evaluating the antimicrobial activity of AMPs. Finally, the applications of AMPs in agriculture, animal husbandry, food industry, and medicine were proposed. We believe that the development of novel AMPs will have a positive impact on human life and health.

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