https://doi.org/10.31925/farmacia.2022.6.1 **REVIEW**

ANTIMICROBIAL PEPTIDES AS POTENTIAL THERAPEUTICS: ADVANTAGES, CHALLENGES AND RECENT ADVANCES

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Abstract

The increasing use of conventional antibiotics and the slow development of new classes of antimicrobials have contributed to the emergence of antimicrobial resistance. This issue has alarmed public health authorities worldwide and has generated a global call for the development of new effective antimicrobial agents. The development of antimicrobial peptides (AMPs) as a novel class of effective therapeutic antimicrobials has attracted widespread attention due to their unique properties. Compared to conventional antibiotics, antimicrobial peptides offer a variety of advantages over their conventional counterparts. These include their potent broad-spectrum antimicrobial activity in addition to their ability to slow the emergence of resistance and their capability of modulating the host immune response. Despite the progress that has been made in the development of antimicrobial peptides, the clinical use of these agents has been hindered by the various inherent structural limitations within AMPs and the stringent pharmaceutical regulatory environment. Accordingly, great efforts have been made to redesign and repurpose AMPs to tackle these challenges and accelerate their arrival at the clinic. This review provides an overview of the various properties of AMPs and comprehensively analyses the various advantages and challenges of employing this class of antimicrobial agents. Finally, it also highlights the recent developments in generating more effective strategies to address the challenges and obstacles hindering the progression of AMPs as effective therapeutic agents.

Rezumat

Utilizarea tot mai frecventă a antibioticelor convenționale și dezvoltarea lentă a noilor clase de antimicrobiene au contribuit la apariția mecanismelor de rezistență. Această problemă a alertat autoritățile de sănătate publică din întreaga lume și a generat nevoia de a dezvolta noi agenți antimicrobieni eficienți. Dezvoltarea peptidelor antimicrobiene (AMP) eficiente a atras atenția pe scară largă datorită proprietăților lor unice. În comparație cu antibioticele convenționale, peptidele antimicrobiene oferă o varietate de avantaje. Printre acestea se numără puternica lor activitate antimicrobiană, capacitatea de a încetini apariția rezistenței și proprietatea de a modula răspunsul imun al gazdei. Totuși, dezvoltarea clinică a acestor agenți a fost împiedicată de limitările structurale inerente din cadrul AMP-urilor, precum și de reglementările farmaceutice. În consecință, acestea sunt reproiectate și reconfigurate pentru a accelera utilizarea lor în clinică. Studiul oferă o imagine de ansamblu a diverselor proprietăți ale AMP-urilor și oferă o analiză cuprinzătoare a diverselor avantaje și provocări ale utilizării acestei clase de agenți antimicrobieni. Se evidențiază, de asemenea, evoluțiile recente în generarea unor strategii mai eficiente pentru a aborda provocările și obstacolele care împiedică progresul AMP-urilor ca agenți terapeutici antimicrobieni.

Keywords: antimicrobial peptides; drug resistance; anti-bacterial agents; peptides; pharmaceutical design; infectious diseases

Introduction

The introduction of antibiotics during the twentieth century marked a new medical era. However, the rise of antibiotic-resistant bacteria has threatened to jeopardize modern medicine's future [1, 2]. Anti-microbial resistance is considered one of the biggest threats to public health, and according to a report released by the CDC in 2019, around 2.8 million people in the US were infected with bacteria that were resistant to antibiotics, and over 35,000 people die annually due to these infections [3]. The World health organization (WHO) warned that the development of multi-drug resistance bacteria could lead to the deaths of up to 10 million people by 2050 [4]. Without immediate action, this issue could become a significant global health dilemma. Although various factors can contribute to the development of antibiotic-resistant bacteria, one of the most common factors that can accelerate this mechanism is the overuse and misuse of antibiotics [5]. In addition to the overuse and misuse of antibiotics, the development of new classes of antimicrobial agents has also been hindered by the slow pace of their development [6]. This issue is referred to as the antibiotic discovery void since the last representative of a novel class of antibiotics to enter the clinic was daptomycin which the FDA approved in the 1980s of the previous century [7]. Daptomycin is a cyclic lipopeptide that was isolated from the actinomycete *Streptomyces roseosporus* and selectively targets only Gram-positive bacteria [8]. For gram-negative pathogens, the situation is even more

difficult as no new antibiotics capable of targeting Gram-negative bacteria have been approved in the past 50 years [9]. In order to prevent humans from entering a post-antibiotic era, the need for effective antimicrobial development research programs is becoming more critical. This is especially true since major pharmaceutical companies have recently withdrawn and suspended their antibiotic research programs [10]. Accordingly, a comprehensive global effort is needed to develop effective, novel classes of antimicrobial agents.

Over the past three decades, the development of antimicrobial peptides (AMPs) has been regarded as one of the most critical factors that have contributed to the search for new antibiotics [11-13]. AMPs are a class of potent broad-spectrum antimicrobials capable of killing bacteria through a membrane-active mechanism [14]. Unlike other antibiotics, they do not require site-specific binding to trigger their activity. Antimicrobial peptides have various advantages over existing antibiotics, such as their ability to slow down the emergence of resistance and their ability to modulate the host immune response [15]. Despite the various mechanisms by which bacteria can develop resistance, the membrane-active antimicrobial system adopted by AMPs is still very challenging and evolutionary burdensome for bacteria to modify in order to acquire resistance [16]. Although their interactions with microbes have been known for millions of years, bacteria have not been able to develop widespread resistance against AMPs, this suggests that AMPs' unique mechanism of action can prevent the development of resistance easily [17]. AMPs typically contain less than 100 amino acids and they are also composed of a significant portion of hydrophobic and positively charged residues, making them ideal for directly targeting bacterial cell membranes [18]. Though AMPs hold great potential in antimicrobial drug development, they still have several undesirable properties for clinical application. Natural AMPs are usually unstable in the gastrointestinal tract and other body fluids; they suffer from poor absorption and distribution accompanied by fast metabolic degradation and excretion, which results in their low bioavailability [19]. Additionally, their flexible structures can also induce interactions with other cellular components, leading to the emergence of various side effects [20]. AMPs also suffer from high production costs and susceptibility to various proteases [21].

Various strategies have been proposed to overcome these challenges, such as using bioengineering, chemical modifications, and developing innovative AMPs delivery systems [22]. With the help of these methods, the production of potent and stable AMPs could be achieved to produce AMPs that are cheaper and potentially capable of reaching the clinic. This review aims to provide a comprehensive overview of the various properties of AMPs and their applications as therapeutic agents. Additionally, this review aims to comprehensively analyse the various advantages and challenges of using this class of antimicrobial agents against bacteria. Finally, it also highlights the recent developments of more effective strategies to address the challenges and obstacles hindering the progression of AMPs as effective therapeutic agents.

Classification of AMPs

AMPs are widely found in different life forms, including animals, plants, fish, and amphibians. They play a role in developing and maintaining an innate immune system designed to protect most living organisms from infection [23]. AMPs are short peptides that contain up to 50 amino acids. Although their structures, sequences, and lengths vary, they have some common features. Most AMPs are composed of arginine, histidine, and lysine and despite the diversity of AMPs that have been discovered so far, most of them share two main features and these include their amphiphilic and net positive charge structures [24]. Additionally, most AMPs have both cationic and hydrophobic amino acid residues that can easily be transversed into membrane-associated patches upon attachment to bacterial membranes, which is responsible for the membrane-induced mechanism of action attributed to AMPs [25].

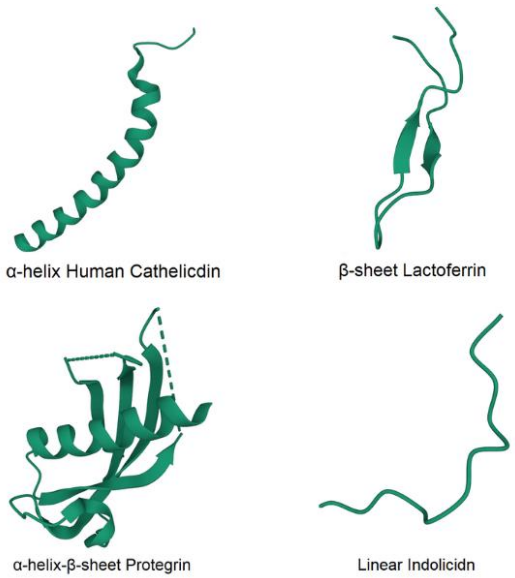

Figure 1.
Secondary structure of AMPs: α-helix (Human Cathelicidin, PDB:2K60), β=sheet (Lactoferrin, PDB: 1LFC), α-helix-β-sheet (Protegrin, PDB: 1KW1), Linear (Indolicidin, PDB: 1G89)
The images were generated and adapted freely from RCSB PDB (Research Collaboratory for Structural Bioinformatics Protein Data Bank) (Website: https://www.rcsb.org)

Due to the immense number of AMPs that have been discovered so far, it is difficult to classify them into systematic categories. However, they can be broadly classified based on their secondary structure [26]. Accordingly, AMPs are categorized into four families, and these include the α-helix, β-sheet, the combined α-helix-β-sheet peptides, linear AMPs, and the more complex cyclic AMPs (Figure. 1). Most α-helical peptides assume random conformation in solution and undergo a radical change in their structure when they interact with bacterial membranes or membrane mimetics and adopt a stable α-helical structure accordingly [27, 28]. On the other hand, β sheets or α-helix-β-sheet peptides have a rigid amphipathic structure that is stabilized by macrocyclic or intra-molecular disulphide bonds [29]. Some AMPs, on the other hand, exhibit a random or extended structure and do not display a specific secondary structure.

Mechanism of Action of AMPs

As part of the innate immune system's first line of defence, many AMPs have evolved over time to be able to kill bacteria without causing toxicity to the host [23]. This is due to the cell surface structure differences between prokaryotic and eukaryotic cells. For Gram-negative bacteria, the outer membrane is arranged so that it contains a high concentration of negatively charged lipid heads [30]. These head groups include phosphatidylglycerol and the lipopoly-saccharide layer [31]. Gram-positive bacteria lack the outer membrane found in Gram-negative bacteria, but contain an enriched thick peptidoglycan layer within the bacterial cell wall that is covered with negatively charged teichoic acids and is arranged to face the inner layer of the Gram-positive bacterial membrane [32]. The inner membrane of Gram-positive organisms contains a higher concentration of negatively charged lipid heads than that of Gram-negative bacteria. (40 - 50% *vs*. ~20%, respectively) [33]. On the other hand, the outer leaflet of a mammalian cell is composed of lipid molecules with zero net charges, such as cholesterol and phosphatidyl-choline, while the inner leaflet is composed of negatively charged lipid heads, such as phosphatidylserine [34]. Accordingly, cationic AMPs can preferentially adsorb to the surface of both Gram-positive and Gram-negative bacterial cells through electrostatic attraction that is driven by the charge differences between the two systems [35]. This is followed by AMPs' ability to permeate through bacterial membranes assisted by their hydrophobic domains leading to membrane instability and rupture and, accordingly, bacterial cell death [36].

Different mechanistic models were proposed to explain the membrane permeation activity of AMPs, and these include the barrel-stave, toroidal pore, and carpet models (Figure. 2) [37].

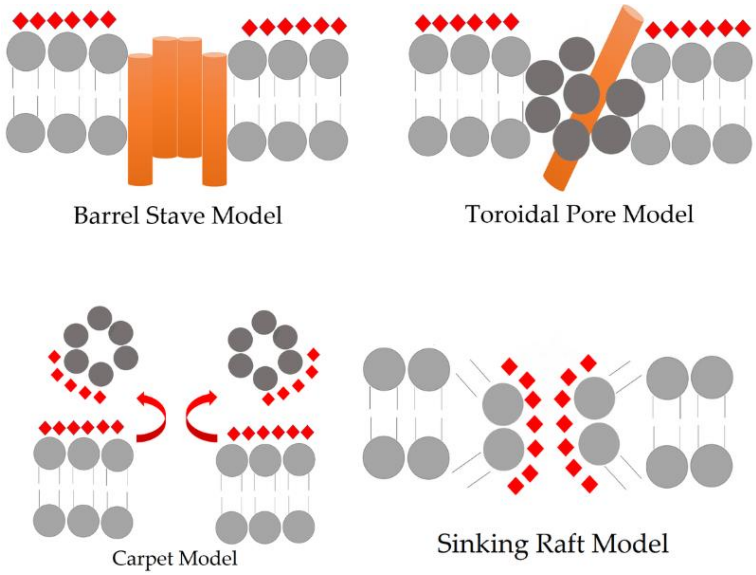

Figure 2.

The different proposed mechanistic models for the activity of AMPs against bacterial cell membranes

The red triangles represent the AMP while the orange tubes represent the pores formed within the bacterial membrane bilayer

All these models assume that the AMPs undergo a facially amphiphilic movement when they interact with the bacterial membranes [38]. It could be argued that all models described are realistic, depending on the AMP examined, as different peptides might fit into a particular model mentioned. Regardless of which actual model describes a peptide's mode of action, the first step for each antimicrobial peptide to display antimicrobial activity is electrostatic interaction with the negatively charged phospholipid head groups

of the lipid membranes [39]. The peptide must then be capable of penetrating the LPS layer that covers the walls of the Gram-negative bacteria or the acidic polysaccharide layer that covers the Gram-positive bacteria [40]. It is after this stage that peptides are able to reach the cytoplasmic membranes, driven by both electrostatic and hydrophobic interactions, and initiate the disruptive membrane mechanisms, which are an intrinsic part of all of the models proposed previously and will be detailed above and in Figure 2.

The barrel stave model

In the barrel stave model, AMPs orient themselves perpendicular to the plane of the membrane and align so that their hydrophobic side chains are exposed to the outer layer, which is hydrophobic in nature, while their hydrophilic regions align themselves inward facing the formed transmembrane pores. These inward parts of the peptides can create consistent channels hence the term "barrel", while the outer parts are termed the "staves" [41]. The formation of these pores will eventually lead to cell lysis by causing leakage of the intracellular components and eventually causing cell death [42].

The Toroidal Pore Model

In the toroidal pore model, the peptides behave in a way similar to those in the barrel stave model in that they align themselves perpendicular to the membrane so they can form pores, but the main difference is that, unlike the barrel stave model, the peptides connect the lipid parts of the membrane and outer layer causing the membrane to face towards the pore. This confirmation will cause a significant strain on the membrane, allowing it to collapse [43, 44].

The carpet model

In the carpet model, AMPs aggregate on the surface of the membranes parallel to the lipid bilayer and coat the membrane like a carpet until they reach a certain threshold concentration at which they cause disturbances in the membrane stability and act like detergents forming cracks and patches that eventually become pores that resemble micelles. This would consequently lead to membrane disintegration, intracellular component leakage, and cell death [45].

Other Models

Several other pore formation models have been proposed, such as the sinking raft model and molecular electroporation (Figure 2). The former involves the accumulation of cationic AMPs on the outer membrane of bacteria. In the latter, the formation of nanopores is triggered by electroporation. The molecular electroporation model only occurs when the charged peptides have a sufficiently high charge density sufficient to reach a membrane electrostatic potential equal to 0.2 V and above [46-47].

The sinking raft model involves the accumulation of AMPs on the outer leaflet of the membrane. This mass imbalance causes a curvature gradient along the membrane, which allows the peptides to sink into it and create transient pores capable of inducing intracellular leakage and cell death [48]. Recent studies have shown that AMPs can potentially act on intracellular bacterial targets and organelles. For instance, magainin II and buforin II were found to bind to DNA and interfere with its replication [49, 50]. Other AMPs, such as indolicidin and pleurocidin were also found to hinder the activity of enzymes involved in the DNA repair and the protein synthesis process [51, 52]. It is worth noting that AMPs do not rely on one mechanism to protect the host or display antimicrobial activity. AMPs act on bacterial targets through a variety of mechanisms during an active infection. The low resistance rate of these peptides to bacterial infections is attributed to their multi-action nature, which could explain their co-existence with bacteria for millions of years [53].

Structural determinants of antimicrobial activity

Despite the various safety and pharmacological concerns associated with the clinical translation of natural AMPs, structure-activity relationship (SAR) studies performed on these antimicrobial agents have revealed several physicochemical parameters that dictate their antimicrobial activity and could be utilized to design novel synthetic AMPs with enhanced properties. Therefore, this section aims to provide a comprehensive analysis of the various parameters that determine the activity and toxicity of AMPs (Figure 3).

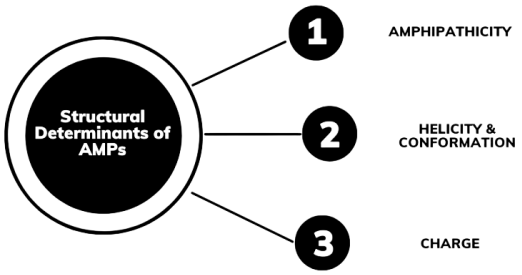

Figure 3.
Structural Determinants of Antimicrobial peptides' activity and toxicity towards both bacterial and mammalian cells

Amphipathicity

One of the most critical factors determining the activity and selectivity of AMPs is amphipathicity, and accordingly, it is important to fine-tune amphipathicity to achieve targeted optimal performance for AMPs. Amphipathicity is a sum of the polarization of the hydrophilic and hydrophobic domains in a peptide, and one measure of amphipathicity is the hydrophobic moment which is a measure of the hydrophobicities of all the individual amino acid residues in a peptide [54]. Increasing the hydrophobic moment would result in increasing the permeabilizing membrane activity of the peptide involved, but with higher

activity towards neutral zwitterionic membranes and with a less profound effect on the negatively –charged bacterial membranes [55]; thus, it could be used as a good measure of the toxicity of peptides and their differential selectivity towards mammalian and bacterial membranes. In the early works of Palermo and Kuroda, they developed a series of random copolymers that exhibited excellent anti-bacterial properties. They demonstrated that even non-peptide systems with random sequences could kill bacteria when introduced to the structure if amphipathicity is properly maintained within the random structure of polymers [56-58]. This clearly displays the importance on amphipathicity in achieving effective antimicrobial activity for AMPs Additionally, their work showed that a gradual increase in hydrophobicity caused the antimicrobial and haemolytic properties of AMPs to increase while an overly hydrophobic structure eventually leading to decreased activity and haemolysis. Other works have demonstrated that amphipathicity cannot be considered to contribute singularly to the overall antimicrobial activity and toxicity of AMPs and, accordingly, it is considered as one of several structural determinants of the overall behaviour of AMPs [59]. Edwards *et al.* have shown that a general increase in amphipathicity is correlated with an increase in antimicrobial activity and toxicity in a series of β-hairpin in-house designed AMPs [60]. They concluded that achieving optimal AMP design requires a delicate balance between all structural determinants involved in the overall behaviour of AMPs and that relying on one structural parameter is not sufficient for the prediction of AMPs behaviour.

Helicity and conformation

The conformation, or three-dimensional topological structure, is a significant structural determinant that contributes to the activity and toxicity of AMPs. Although diverse in structure, most AMPs fall into two major groups: the α-helical and the β-sheet peptides. Others fall into the smaller groups of the previously described combined α-helix-β-sheet peptides, and the extended and highly complex peptides, which vary significantly from the previous two. Regarding their activity, these conformations are of substantial importance as they are thought to be involved in pore formation inside the bacterial cytoplasmic membranes following their initial interaction. Any alteration in their structure could lead to loss of activity or drastically increase their toxicity [61]. Helicity is considered a major indicator of effective antimicrobial activity, and increased peptide helicity has been reported to significantly increase antimicrobial activity [62]. Yoko *et al.* utilized helix-destabilizing sarcosine to study the effect of helicity on the overall anti-microbial activity of AMPs. Their work revealed that destabilizing the helical structure of several AMPs decreased both antimicrobial activity and increased cell cytotoxicity [63].

Charge

The presence of positively-charged residues within the amino acid sequence of AMPs is considered crucial to their activity as bacterial membranes are composed of negatively-charged acidic phospholipids. These, and the presence of the LPS and teichoic acids in the outer membrane of Gram-positive and Gram-negative bacteria [64], are responsible for the initial electrostatic interaction and the accumulation of these peptides on the surfaces of bacterial membranes that ultimately leads to cell lysis and destruction. As with all other factors, there is a charge range for an optimum activity to be achieved and that is reported to be from (+2 to +6), as AMPs with a lower charge than this range would have no antimicrobial activity at all, and a higher charge more than +6 could also have negative effects on the activity as it is thought to lead to excessive membrane binding which could impede the translocation of the peptides through the membranes. Different studies have demonstrated that the charge type and its density had a significant influence on the activity and toxicity of AMPs [65]. Additionally, the charge is one of the structural determinants of AMPs that are positively and negatively influenced by the pH of their surrounding environment. At acidic pH (4 - 6), AMPs' antimicrobial activity could be enhanced as low pH can cause the protonation of histidine, aspartic, and glutamic acid thus increasing the charge of the peptides. On the other hand, higher pH and salt concentrations can negatively impact the antimicrobial activity of AMPs [66, 67].

Disadvantages of AMPs

AMPs face several limitations for effective clinical development and these include their high mammalian cell cytotoxicity and lack of consistent pharmacokinetic profiles [68]. AMPs also suffer from high production costs and a lack of resistance against proteases [69]. The *in vitro* MIC values of AMPs and their correlated *in vivo* activity can also be affected by AMPs' loss of activity due to plasma protein binding or physiological salt concentrations [70]. These issues combined can lead to different and unpredictable clinical outcomes during their clinical development program. Therefore, several strategies have been identified to improve the performance of AMPs towards clinical development.

In vivo Efficacy

One of the main obstacles to the clinical development of novel AMPs is the lack of a sufficient activity match between their *in vitro* and *in vivo* efficacy [71]. Despite the potent antimicrobial activity of AMPs *in vitro*, their *in vivo* performance was below expectations. This issue is apparent in the failure of several major novel and promising AMPs, such as iseganan, omiganan, pexiganan, and surotomycin to pass phase III trials [72]. The weak *in vivo* performance

of AMPs is also attributed to the same structural and physicochemical parameters that allow AMPs to display such potent antimicrobial activity *in vitro*. In general, most AMPs are designed to adopt a facially amphipathic structure; this allows them to maintain their hydrophobic face while separating the hydrophilic and cationic surfaces accordingly [73]. Although the amphipathic structure is crucial for bacterial membrane disruption, it also causes non-specific cellular and systemic interactions. For instance, many biomolecules, such as DNA, glucose, and serum proteins, are negatively charged within the human body and could be an off-side target for AMPs when administered *in vivo* [74]. Unfortunately, the non-specific binding of cationic AMPs to these negatively charged biomolecules *in vivo* can significantly limit their effective antimicrobial concentrations against target pathogens [75]. The hydrophobic surface of AMPs can also induce non-specific cell surface binding and aggregation. As most of these AMPs are derived from natural L-amino acid-based sequences, they are also vulnerable to degradation and attack by systemic proteases [76]. Due to their sensitivity to environmental conditions, many of the antimicrobial activities of AMPs are lost when exposed to salt, pH, and serum [77, 78].

Resistance

Various studies have shown that developing resistance against AMPs is possible and could be triggered by membrane surface charge modifications [79]. Additionally, bacteria can secrete different proteases to neutralize and cleave AMPs rendering them ineffective [80]. Finally, recent studies suggested that bacteria can develop or employ some of its existing efflux pumps to decrease the bacterial intracellular concentrations of AMPs [81]. Although the development of resistance against AMPs is possible, it is not considered a critical issue when compared to conventional antibiotics. This is mainly attributed to the complexity of the membrane structural alterations needed by bacteria to acquire resistant properties against AMPs [82]. It is typically not feasible for microbes to maintain and achieve this acquired resistance without compromising their membrane structural and functional integrity.

Stability

AMPs also suffer from low *in vivo* stability. They can be easily degraded by proteolytic enzymes, which are present in the digestive and intestinal tract [83]. Because of this, topical administration is often the preferred delivery method for AMPs. Chemical modifications and other innovative and novel delivery systems can improve their stability. The issue of *in vivo* stability would constitute a major issue for AMP's development for intravenous and oral formulations. These formulations would suffer from a very short half-life due to the abundant number of proteases found in serum and the digestive tract [84]. Attempts to bypass this challenge are to design AMP formulations for topical or intramuscular administration or by introducing chemical modification strategies to alter the peptide template to resist protease degradation [85].

Toxicity

AMPs can induce toxicity at different levels including cellular and systemic toxicity [86]. The cellular toxicity is usually caused by the same amphipathic structure that endows AMPs with their antimicrobial activity. Despite the presence of less negatively charged lipid molecules in the outer leaflet of mammalian cell membranes when in comparison with their bacterial counterparts, it still contains significant amounts of negatively charged polysaccharides and glycoproteins [87]. This can cause AMPs to accumulate on mammalian cell membranes causing cellular disruption and consequently inducing cellular toxicity.

Additionally, AMPs can induce significant cellular haemolysis when their concentration passes a certain threshold, and thus this endows the relevant AMP with a very narrow therapeutic index that can hinder their clinical application [88]. AMPs can interact with other cell surface receptors and disrupt normal signalling pathways [89]. Additionally, some AMPs can cross the cellular membrane and interact with other cellular components, such as the DNA and intracellular organelles which can prevent the synthesis of different proteins and could trigger programmed cell death or what is known as apoptosis [49]. The exact mechanism by which AMPs can cause systemic toxicity is not well known. In addition to their toxic cellular effects, they can also interact with other tissues and organs [90]. Some of the common systemic toxic effects include interference with the central nervous system due to the ability of some AMPs to cross the blood-brain barrier, in addition to inducing blood vessel blockage and nephrotoxicity [91].

Accordingly, most clinical trials designed to target different human infections are focused on the topical application of AMPs. This strategy ensures that AMPs are not able to trigger unpredicted systemic toxicity. Additionally, topical application of AMPs can also increase their effective local concentration and make them less susceptible to proteases.

Cost

The lack of financial resources and investment in antimicrobial drug development has significantly contributed to the slow development of AMPs [92]. Currently, many clinically available antibiotics can still be employed to treat several infections, even the ones caused by highly resistant Gram-positive and Gram-negative bacteria, and these include antibiotics such as fluoroquinolones and colistin. Additionally, due to the high cost of developing new antibiotics and the competition from existing drugs, the profit margins of newly developed

compounds are typically not attractive to the pharmaceutical industry [93]. Instead of undergoing costly and time-consuming clinical and preclinical studies, many pharmaceutical companies are now developing antibiotic derivatives or combinations of existing commercial antimicrobials with known safety and PD/PK profiles instead of embarking on innovative antimicrobial research and development programs. In addition, the cost of producing amino acid-based peptides such as AMPs is significantly higher than that of conventional antibiotics [94]. This is due to the high cost of the industrial technical requirements needed to produce AMPs by solid phase chemistry, which is significantly more expensive than conventional methods. Reducing the costs by developing shorter peptides or using fermentation or biotechnological engineering is desirable to resolve this issue.

Strategies to enhance the properties of AMPs

In order to improve the stability and safety of AMPs, synthetic approaches were pursued using natural AMPs as templates for antimicrobial drug development during the last two decades [95]. These efforts were mainly focused on mutating and modifying one or more amino acid residues in order to enhance the properties of natural AMPs and create new and improved de novo synthetic AMP templates to enhance the properties and avoid some of the drawbacks of AMPs mentioned previously. The development of a better understanding of the SAR of AMPs led to the creation of a wide range of synthetic peptides, peptoids, oligomers, polymers, and peptidomimetics during the first decade of the 21st century [95-97]. These studies have greatly improved our understanding of the properties of AMPs and resulted in the development of many promising AMP-based antimicrobial drug candidates. The following section will detail some of the innovations and technological advancements that were pursued in the recent decade to tackle the disadvantages of AMPs mentioned previously.

Chemically Modified Synthetic AMPs

One of the most common strategies employed to improve the efficiency and effectiveness of natural AMPs is by modifying their amino acid sequences to substitute the original ones with proteinogenic counterparts [98]. This method has proved to be very effective in either enhancing the antimicrobial activity or the stability of the AMP *in vivo*. One example is pexiganan, a synthetic version of natural AMP magainin 2 developed through single-site amino acid substitutions and related SAR studies to enhance its selectivity and activity towards microbial cells. Pexiganan was also stabilized against tissue-secreted proteases [99]. The modified pexiganan exhibited broad-spectrum activity against different types of bacteria, including those resistant to antibiotics [100]. Due to its excellent *in vitro* and *in vivo* properties, the peptide was advanced to clinical trials and was evaluated for the treatment of diabetic foot ulcers in two clinical phase III trials [101]. This strategy has proven to be very useful in upgrading natural AMPs and allowed several synthetic versions of natural AMPs to reach the late stages of their clinical trials. Several examples of such synthetic versions of original natural peptides include iseganan, omiganan and p113, which were developed based on the natural template sequence of histatin, indolicidin and protegrin, respectively [102-104]. Compared to natural AMPs, synthetic peptides are more stable and exhibit better selectivity and activity toward bacterial cells. However, significant time and labour are still required to develop these molecules as their synthesis is laboursome and expensive. Although substituting certain amino acid residues with other ones in AMPs does not result in the loss of antimicrobial activity, it does imply that the specific amino acid sequence is not required for the activity of these molecules. It is also believed that the overall physicochemical properties of these compounds are more crucial in determining their toxicity and activity.

Antimicrobial peptide oligomers and polymers

The development of synthetic antimicrobial polymers and oligomers allowed researchers to mimic the physicochemical properties of AMPs while reducing their manufacturing costs significantly [105]. Antimicrobial oligomers are usually defined as polydispersed molecules with a molecular weight ranging from 100 - 3000 Daltons, while antimicrobial polymers usually have a molecular weight larger than 3000 Da [106]. Polymeric peptides are known to be very advantageous compared to their synthetic peptide or natural counterparts, which is attributed to their ability to be manufactured in a few synthetic steps [107]. Additionally, and due to their non-natural origin, antimicrobial polymers are known to exhibit excellent resistance to various environmental conditions such as protease degradation and environmental stress [108]. Moreover, and due to the availability of their basic building blocks, the cost of producing these compounds has also been considered highly economically feasible. Examples of such oligomers and polymers include polymers of methacrylamide, polymethacrylate and nylon-3 [109-111].

The incorporation of D-Amino acids

Due to the unstable nature of natural AMPs against proteases, their clinical applications have been limited as they suffer from *in vivo* protease-induced degradation. To overcome AMPs' enzymatic susceptibility, several attempts have been carried out to incorporate D-amino acids at the protease cleavage site within the peptide sequence of AMPs rather than using natural ones to avoid this limitation.

This method managed to enhance the stability of these peptides and accordingly increase their antimicrobial activity. In some cases, this method promoted the potency of the peptides by significantly lowering their MIC values. For example, W3R6 (4) was completely transformed into an all-D-enantiomer, and the substitution increased the parent peptide's stability significantly with a slight decrease in activity and negligible toxicity [112]. Another linear peptide, DMPC-10A, which is a 10-mer peptide that was modified by substituting all L-Lys and L-Leu with their respective D-form amino acid residues in order to prevent its enzymatic degradation, proved to be equally effective to the parent peptide regarding its antimicrobial potency and displayed lower cytotoxicity [113].

Sidechain modification

One of the most common strategies used to improve the stability of AMPs is by chemical modification of their respective side chains. This method can improve the susceptibility of the peptides to proteolysis. However, in terms of their antimicrobial activity, this approach seems to have little influence on the potency. Adding N-methylated residues to AMPs can help improve their stability against the effects of enzymatic degradation [114, 115]. It can also increase its hydrophobicity and promote antimicrobial activity. Although the acetylation of N-terminal residues within AMPs can improve the stability and helical content of the peptide, it can also reduce the net positive charge of AMPs [116]. Additionally, this modification can increase peptide-based membrane permeation and can negatively affect the selectivity of AMPs towards bacterial membranes [117]. On the other hand, C-terminal capping can improve AMPs' stability through carboxyl-mediated modifications, particularly against metabolic degradation [118]. The end modifications at the N-terminus, including acetylation, were successfully employed to enhance the stability of AMPs. One example is the AMP L163, the peptide N-terminal acetylation enhanced the stability of the peptide against pH, plasma and protease degradation. Additionally, the acetylation enhanced the antimicrobial activity of the peptide against multidrug-resistant strains of both Gram-positive and Gram-negative bacteria [119].

Delivery

A wide range of delivery systems has been developed to overcome the toxicity and stability issues of AMPs. These include systems designed to covalently attach to the AMP or as a formulation capable of encapsulating and shielding the AMP using lipids or other polymers that would allow AMPs nanoencapsulation. Several delivery systems can be employed to release active AMPs based on the environmental conditions such as pH, salt concentration and *in vivo* enzymes. One such example is an ultrashort penta AMP (RBRBR) that was encapsulated into chitosan-based nanoparticles [120]. The nanoencapsulation managed to maintain the peptide's antimicrobial activity, while eliminating its toxicity and enhancing its stability. Rodríguez López *et al*. have recently developed a chitosan/ hyaluronic acid-coated titanium surface for the delivery of beta-sheet peptide mimetics that inhibited planktonic and biofilm growth of bacteria and managed to form a sustained release delivery system [121].

Ultrashort Antimicrobial Peptides (USAMPs)

Most natural AMPs are in the 10 to 50 amino acid range. The length of these compounds directly affects the production cost of these chemicals during the development stage. To minimize this cost, the development of new ultrashort and/or truncated versions of these compounds has been pursued. Works performed by Almaaytah *et al*. demonstrated the capability of designing USAMPs consisting of 4 - 6 amino acids with significant antimicrobial potency and negligible toxicity, the peptides were also conjugated to other antimicrobial chemical sidechains to enhance the antibacterial activity [122, 123]. Their work has also explored the potential synergism of USAMPs with conventional antibiotics and has consistently demonstrated that combining AMPs with antibiotics and in particular with levofloxacin could decrease the minimal active concentrations of the AMPs to a nanomolar level which would reduce both the toxicity and cost of AMPs.

Conclusions and future perspectives

AMPs have been identified as promising candidates for developing new therapeutic agents. Several studies have recently shown that combining various antimicrobial peptides with conventional antibiotics can increase their potency and reduce the side effects of conventional therapy. This combination can also help decrease the risk of drug resistance and improve the effective therapeutic dose. The interest in the development of antimicrobial peptides has been proliferating recently. Due to their broad-spectrum activity and low resistance emergence, they have attracted the interest of industry and academia. However, their high production costs and poor pharmacokinetic profiles have prevented them from being fully utilized. Despite the main technical challenges that AMPs face, several recent technological advancements that are addressed these technical barriers have allowed AMPs to be again considered promising antimicrobial drug candidates and are in the state of commercialization by several pharmaceutical industries. Since daptomycin was approved in the 1980s, no new AMPs have been approved. However, in the near future, multiple AMPs are expected to be used as a combination therapy in clinical settings.

Conflict of interest

The authors declare no conflict of interest.

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