Molecular Biology Reports
https://doi.org/10.1007/s11033-022-07405-1

REVIEW

# Anti-Microbial Peptides: Strategies of Design and Development and Their Promising Wound-Healing Activities

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Received: 22 January 2022 / Accepted: 17 March 2022


## Abstract

**Background** Current approaches used to overcome microbial infections are becoming inefficient due to the overuse or misuse of antibiotics. Antimicrobial peptides (AMPs) are one of the most promising substitutional candidates for commercial antibiotics.

**Methods and Results** The publications in the field of *in silico* design of AMPs with focus on the wound-healing AMPs were searched though SCOPUS and PubMed. Through publications, it was reported that a number of AMPs approved for clinical use have also showed efficient wound-healing activity. Todays, the design and production of synthetic types of AMPs have attracted attention in order to expand their applications and to cope with their limitations and adverse effects. In this paper, the currently published researches in the field of AMPs and their wound-healing features were summarized and the strategies used in AMPs design and development have been reviewed. Moreover, different databases and online servers used in this regard were summarized.

**Conclusion** Design and development of active AMPs, especially those with wound-healing activity, can be performed using bioinformatics servers and software, regarding AMPs structure-activity relationships.

**Keywords** Anti-microbial peptides · Wound-healing · Design · *In-silico* · Structure-activity relationship

## Introduction

In the past several decades, microbial infections are a great warning to global health because of the misuse/overuse of antibiotics that has resulted in multidrug resistance (MDR) [1–4]. MDR bacteria have led to a rise in nosocomial infections and in-hospital mortality [5]. In addition, due to economic and regulatory barriers, developing new antibiotic products in the pharmaceutical industry goes forward at a slow speed. Therefore, the conditions for coping with antibiotic resistance and infections caused by MDR bacteria are seriously complex [5, 6].

In these contexts, antimicrobial peptides (AMPs), a group of evolutionarily preserved molecules that play vital roles within the innate system in all live organisms, are auspicious choices to deal with bacterial infections and manage microbial spreading [3, 7, 8]. These are usually small, cationic, and amphiphilic molecules [9, 10]. While most antimicrobial peptides are cationic, some anionic antimicrobial peptides have been found in several organisms, playing a significant role in the innate immune defense [11, 12]. AMPs show a wide range of bioactivities, including anti-methicillin-resistant *Staphylococcus aureus* (MRSA), anti-tuberculosis, anti-sepsis, anti-toxin, antiviral, anti-HIV, antifungal, anti-parasitic, anticancer, anti-diabetic, wound healing and anti-inflammatory activities [7, 13]. Figure 1 summarized several functions of AMPs.

While these features appear to be promising for therapeutic development, AMPs possess a number of drawbacks, including chemical and physical instability [14], decreased activity in the presence of high salt conditions, divalent cations, changeable pH [15], simple *in vivo* enzymatic

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Published online: 08 May 2022

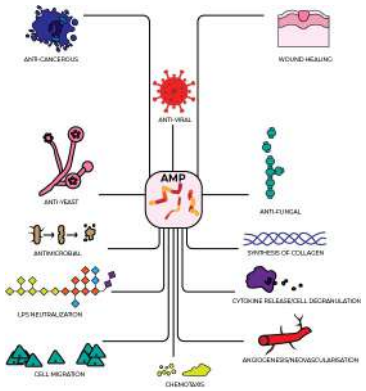


**Fig. 1** A schematic diagram of diverse activity of AMPs. AMPs show a wide range of bioactivities such as anti-cancerous, anti-viral, anti-fungal, and anti-yeast, wound-healing, LPS neutralization, cell migration, angiogenesis, and neovascularization. In addition, they seem to play some roles in cytokine release, cell degranulation, and the synthesis of collagen. LPS: lipopolysaccharide

digestion, comparatively poor activity compared to antibiotics, toxicity to eukaryotic cells, and finally expensive production costs [16]. These flaws have encouraged the advance of synthetic peptides imitating the function of natural ones. Optimized sequences of the engineered peptides have demonstrated increased stability and potency across a broader range of physiological conditions [17]. Nevertheless, to overcome these shortcomings, there needs much exertion to design new AMPs. Investigation into AMPs is constantly progressing, and many bits of knowledge about AMPs have been accumulated in a variety of AMP databases. Therefore, to develop knowledge in the design of antimicrobial peptides with different therapeutic capacities, researchers should perpetually refresh their knowledge on new helpful databases and tools. In this review, basic concepts of AMPs structure and function, classification, the dedicated online web servers, and tools for designing new AMPs have been discussed, with a specific focus on the use of them in wound healing. This manuscript tried to more deeply discussed on the computational design of AMPs for their application in wound healing. Since the use of existing servers and databases is one of researchers' concerns, and a comprehensive article to guide users is not available, in this article, we have tried to address this issue and describe the benefits of the most common servers. In addition, *in vitro* assays to confirm the antimicrobial properties of designed peptides that have been less discussed in other literatures was also considered. Making trails on novel AMPs is an important step to introducing the new peptides as a promising candidate for an alternative to traditional antibiotics, which in addition discussed under a separated subtitle.

## Classification of AMPs

AMPs, also termed host defense peptides (HDPs), are found in all life forms, including fungi, bacteria, insects, plants, and vertebrates. AMPs are typically small peptides with almost fewer than 50 amino acids. They usually have a net charge of + 2 to + 9 in a physiological environment because of containing acidic amino acids, such as lysine and arginine in their sequence. AMPs contain hydrophobic residues by approximately 50%, so they prefer amphipathic conformation when interacting with membranes [7, 18, 19].

These natural peptides differ strikingly in sequence and structural conformation and, in general, are classified into four structural groups based on their conformations: α-helices, β-sheets, mixed structures, and non-α- or β-structures (known as extended form) [18, 20]. Furthermore, AMPs with more complex topologies as well as cyclic peptides are progressively recorded. Magainin, protegrin, LL-37 are prominent examples of alpha-helical AMPs. The lack of cysteine residues in the sequence is a prominent feature of members of this family.

The beta-sheet peptides are composed of at least two β strands with 2 to 4 disulfide bonds between strands and adopt comparatively stiff structures. This class of peptides contains α-defensins, β-defensins, drosomycins, thionins, and plectasin [21].

Some AMPs that do not form any particular 3D structure, either in solution or in contact with membranes, are considered extended linear structures. They have no α-helices or β- sheets and are often rich in one type of amino acid, generally glycine, proline, tryptophan, or histidine. Prominent examples include abaecin from honeybees, bovine indolicidin, human histatins, and hymenoptaecins from various insects [22].

The origin of AMPs can be ribosomal or non-ribosomal. Therefore, they can also be classified based on their biosynthetic mechanism. Gramicidin, polymyxin, bacitracin, daptomycin, bleomycin, cyclosporine, vancomycin, and teicoplanin are examples of non-ribosomal peptides currently used clinically. Although the synthesis of non-ribosomal AMPs is usually constrained to bacteria and fungi, AMPs encoded by nuclear genes exist in all kingdoms of life [22, 23].

Due to expression in all types of organisms, antimicrobial peptides can also be classified based on their source: Mammalian, amphibian-derived, insect-derived, plant-derived, microorganisms-derived, marine organisms-derived, and synthetic AMPs. Cathelicidins along with defensins are the two most common mammalian AMP families while maganinin is the most famous AMP in frogs. The first discovered AMP in the plants is Thionins [24]. Plant defensins are one of the greatest antimicrobial peptide superfamilies that

extensively exist in the plant kingdom [25]. Moreover, the first discovered antimicrobial peptide in insects was Cecropin. It was isolated from the hemolymph of immunized *Hyalophora cecropia pupae* in 1980. Cecropins in *H. cecropia* is categorized into subfamilies A, B, C, D, and E [26]. Another source of AMPs is microorganisms, such as bacteria and fungi. Some well-known peptides-derived bacteria are nisin and gramicidin extracted from *Lactococcus lactis*, *Bacillus subtilis*, and *Bacillus brevis* [27].

Exceptionally, marine origin AMPs make up less than 5% of the more than 2,400 natural and synthetic AMPs discovered, so far. A wide variety of marine invertebrates and fish have been shown to have the bulk of these peptides. These organisms have no specificity and memory in immune response similar to B and T lymphocytes mediated immune memory, thus, AMPs are critical to their survival, as the initial defense line against pathogens. Circulating hemolymph is a rich source of antimicrobial peptides in marine invertebrates [28].

Despite a very high diversity of natural antimicrobial peptides, the limitations of these peptides for use as therapeutic peptides have led to the synthesis of a wide variety of peptides artificially. These peptides are designed and synthesized inspired by natural peptides [29].

## Mechanism of AMPs action

AMPs can act via membranous and non-membranous mechanisms (by interacting with intracellular targets such as DNA, RNA, and proteins) for killing pathogens [30]. They generally work by compromising the cell membrane's integrity, resulting in the rapid release of vesicles and other cellular components. The electrostatic attraction between the bacterial wall with negative charges and AMPs with positive charges is usually the basis of the interaction [31]. When the AMPs bind to the bacterial membrane, a critical concentration is required to proceed. Subsequently, AMPs get access to the phospholipid bilayer membrane by first aggregating, then forming various complex structures (through four different mechanisms, such as the "degenerate," "toroidal pore," "barrel-state," or "carpet" models (Fig. 2)), and lastly penetrating into the membrane and lysing the cell [32, 33].

Besides these mechanisms, AMPs can diffuse through the cell membrane and accumulate inside the cell. Subsequently, they block DNA replication and disrupt RNA and protein synthesis leading to cell wall lysis [34]. For example, proline-rich AMPs can penetrate through bacterial membranes and kill bacteria by blocking protein synthesis [35]. Interfering with protein folding can potentially have antibacterial effects [36]. According to a recent study,

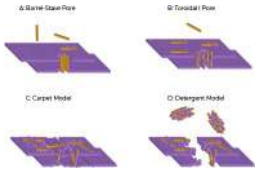

**Fig. 2** Four different models for antimicrobial activity. A and B: In barrel-stave pore and toroidal pore models membrane-spanning aqueous channels are formed. C: In carpet model, peptides permeabilize membranes by carpeting the bilayer. At high concentrations carpet model usually behave like detergent model. D: AMPs at a high concentration act like detergents

some AMPs such as Cathelicidins and Papiliocin can produce reactive oxygen species (ROS) and alter mitochondrial activity, resulting in cell death [7]. Indolicidin, a bovine cathelicidin cationic host defense peptide, exhibits both the disruption of the bacterial membrane and the inhibition of DNA synthesis [37].

## Relationship between structure and function of AMPs

Several structural factors should be consider for understanding the therapeutic potential of AMPs. The substantial structural rearrangements adopted on contact with microbe membranes, as well as the appropriate balance of hydrophobicity, amphipathicity, and cationicity are all included in these criteria [38]. The α-helix is the most prevalent motif in AMPs. Many AMPs, however, adopt extended or unstructured conformation and just during interaction with phospholipid membranes exhibit distinct α-helical- or β-sheet-like [39]. Because the presence of the alpha-helix topology is critical for enhancing membrane interaction, for aiding cell lysis, and antimicrobial activity in general, some residues are dynamically involved in the design of new peptides and undergone some modifications in the peptide sequences. Because antibacterial efficacy correlates with the generation of a helical conformation in a membranous environment rather than intrinsic helical stability, these modifications are sometimes not only ineffective but can also cause harm in mammalian cells [40]. The net positive charge or positive charge density has a critical limit on a given a-helical antimicrobial peptide. Studies have shown that when approximately 30% of residues are positively charged, they can provide the best therapeutic index. Higher charge density (more than 34% positively charged residues), on the other hand, leads to increased hemolytic activity and a lower therapeutic index. For antimicrobial peptide selectivity toward microbes, a minimum charge threshold, maybe as low as +2, appears to be required [41]. By comparing therapeutic indices in different microorganisms, it

has been determined that a peptide with a net charge of +8 is the best option without affecting hemolytic activity [42].

Hydrophobicity is another crucial factor to consider when determining an AMP's overall activity. It plays a crucial function in the possible interaction with bacterial membranes and may be linked to antibacterial specificity acquisition or loss. The hydrophobicity of AMPs is reduced, resulting in fewer interactions with mammalian cells. At the same time, as long as the peptide has a sufficient positive charge, it is preferred to target bacterial cell membranes [43].

In addition to the factors mentioned, amphipathicity is essential for antimicrobial efficacy because it allows antimicrobial peptides to embed their hydrophobic domain in the bacterial membrane bilayer. The hydrophobic moment, or *MH*, is computed as the vectorial sum of individual amino acid hydrophobicities normalized to an ideal helix and is commonly an indicator used for the examination of the involvement of amphipathicity in peptide antimicrobial activity. [38, 44]. The amphipathicity of peptides with a β-sheet topology is brought about by the arrangement of β-sheets into segregated polar and non-polar faces, which are maintained by disulfide bridges or head-to-tail cyclization and give incredible conformational rigidity in aqueous solution. In this situation, salt-bridges may contribute significantly to the secondary structure's overall stability. Antimicrobial peptides can successfully interact with target membranes thanks to the polar and non-polar domains of β-strands. The amphipathic nature of these molecules allows membrane disruption via the creation of transmembrane channels when they interact with the bacterial cell membrane [38].

The length of the peptides differs significantly amongst AMPs. An amphipathic structure with segregated hydrophobic and hydrophilic domains requires at least seven to eight amino acids. Increases in peptide length result in a corresponding rise in peptide net charge, according to studies. On the contrary, as peptide length increases, the hydrophobic quantity decreases [45]. However, no fixed length of a peptide sequence needs to be considered while designing new antimicrobial peptides [38].

Another determining factor of the whole activity of a given AMP is the polar angle (θp). It is a measurement of a peptide conformed to an amphipathic helix's relative fraction of polar vs. non-polar facets. The polar angle of a hypothetical α-helical peptide with one face entirely made up of hydrophobic residues and the other wholly made up of charged residues would be 180°. The polar angle would be correspondingly reduced if the segregation between these domains was reduced or the hydrophobic ratio of the helix was increased. A lower polar angle (and hence a better hydrophobic surface) in native and synthesized peptides has been linked to a more extraordinary ability to permeabilize membranes in several investigations [41]. In a study, Natsuko Uematsu and Katsumi Matsuzaki investigated the effect of polar angle on the antibacterial action of AMPs. They did this by creating two nearly equivalent peptides in terms of physicochemical features, excluding the polar angle (Peptides with polar angles of 100° and 180°). Compared to θp180, θp100 had a stronger membrane permeabilization potential, a larger flip-flop rate, and more antibacterial activity due to a higher pore creation rate. The peptide translocation rate of θp100 was greater, consistent with these findings. Moreover, p100 pores included fewer peptides than p180 pores, and p100 pores engaged more lipid molecules, as evidenced by its cationic specificity. The polar angle has been a crucial factor in peptide-lipid interactions [46].

## Strategies used in AMPs design and development

### Different methods of design novel AMPs

Nowadays, peptide discovery is accelerated by library screening via both rational and non-rational approaches. Designing new peptides rationally is based on three primary methods: template-based, physicochemical, and *de novo* methods, attaining novel peptides and/or improving existing ones [6].

The purpose of template-based design is to selectively add or eliminate one or more residues or from a well-known peptide sequence. Thus, the position of the residue might change throughout the sequence. These changes usually lead to reduction of the peptide size. This method assists in identifying novel AMP sequences even from inactive peptides. In a study, Yara Al Tall and colleagues developed a peptide with superior antibacterial efficacy than parent peptides, utilizing a pattern-based technique. They designed a hybrid peptide by combining two cathelicidins (LL-37 and BMAP-27). The helicity content of the hybrid peptide was improved through analysis by some online prediction tools. This 25-amino-acid peptide had a lower MIC value than the parental peptides against a wide range of bacteria. Furthermore, the hybrid peptide has anti-biofilm capabilities, nevertheless the parental peptides did not. Hemolytic testing revealed that using this peptide in the concentrations needed to kill bacteria is safer than using the parents; however, it should be highlighted that it must be used with caution as an anti-biofilm peptide because the dosage required to this target is toxic on human cells [47]. Besides combining two or more pattern sequences and designing new sequences, it is also possible to design a peptide library using a highly

useful alignment tool with a sequence similar to a specific natural peptide family. Then, by screening this library using various online algorithms such as $CAMP_{R3}$, one or more peptide sequences with antimicrobial properties can be obtained that may have better bioactivities than the pattern sequence.

The physicochemical design creates analogs with diverse physicochemical characteristics from famous sequences.

**Table 1** Important databases dedicated to AMPs

| Name | Description | Link | Reference |
|---|---|---|---|
| $CAMP_{R3}$ | Database of antimicrobial peptides and proteins | http://www.camp.bicnirrh.res.in/ | [52] |
| ADP | One of the biggest antimicrobial peptide databases | https://aps.unmc.edu/ | [53] |
| $DBAASP_{V.3.0}$ | Database of antimicrobial peptides and proteins | https://dbaasp.org | [54] |
| DRAMP | Database of antimicrobial peptides and proteins | http://dramp.cpu-bioinfor.org/ | [55] |
| ADAPTABLE | Database of antimicrobial peptides and proteins | http://gec.u-picardie.fr/adaptable/ | [56] |
| YADAMP | Database of antimicrobial peptides | http://yadamp.unisa.it/default.aspx | [57] |
| AntiTbPdb | Database of anti-tubercular peptides | http://webs.iiitd.edu.in/raghava/antitbpdb/ | [58] |
| BaAMPs | Database of antimicrobial peptides tested against microbial biofilms | http://www.baamps.it/ | [59] |
| Peptaibol Database | database for sequences and structures of naturally occurring peptaibols | http://peptaibol.cryst.bbk.ac.uk/home.shtml | [60] |
| Biolip | Database of protein-ligand interactions. | https://zhanglab.dcmb.med.umich.edu/BioLiP/ | [61] |
| Cybase | Database of cyclic proteins and peptides with various bioactivities | http://www.cybase.org.au/?page=welcome | [62] |
| DADP | Database of defense peptides. Includes mainly antimicrobial activities involved in biosynthesis | http://split4.pmfst.hr/dadp/ | [63] |
| Norine | database of nonribosomal peptides together with tools for their analysis | https://bioinfo.lifl.fr/norine/ | [64] |
| CancerPPD | Database of anticancer peptides and proteins | https://webs.iiitd.edu.in/raghava/cancerppd/mobile/ | [65] |
| iACP | A sequence-based tool for identifying anticancer peptides | http://lin-group.cn/server/iACP | [66] |
| HEMOLYTIK | Database of Hemolytic and Non-hemolytic Peptides | https://webs.iiitd.edu.in/raghava/hemolytik/ | [67] |
| CPPsite 2.0 | Database of cell penetrating peptides | https://webs.iiitd.edu.in/raghava/cppsite/ | [68] |

**Table 2** Useful online web tools, using for designing or identifying therapeutic peptides

| Name | Description | Link | Reference |
|---|---|---|---|
| PreAIP | An accurate Predictor of Anti-Inflammatory Peptides | http://kurata14.bio.kyutech.ac.jp/PreAIP/ | [69] |
| ExPASy tool, ProtParam. | A tool for the computation of various physical and chemical parameters | https://web.expasy.org/protparam/ | [70] |
| CellPPD | A tool for prediction and designing efficient cell penetrating peptides | https://webs.iiitd.edu.in/raghava/cellppd/index.html | [71] |
| BIPEP | A tool for prediction biofilm inhibitory peptides | http://cbb1.ut.ac.ir/BIPClassifier/Index | [72] |
| HemoPI | A tool for prediction of hemolytic activity of peptides | https://webs.iiitd.edu.in/raghava/hemopi/design.php | [73] |
| ToxinPred | A Tool for prediction and designing toxic/non-toxic peptides | https://webs.iiitd.edu.in/raghava/toxinpred/ | [74] |
| HLP | A web-server for predicting half-life of peptides in intestine like environment | https://webs.iiitd.edu.in/raghava/hlp/ | [75] |
| PeptideCutter | A tool for predicting potential cleavage sites cleaved by proteases or chemicals in a given protein sequence | https://web.expasy.org/peptide_cutter/ | [70] |
| iAMPpred | A web-server for predicting anti-bacterial, anti-fungal and anti-biofilm peptides | http://cabgrid.res.in:8080/amppred/index.html | [76] |
| EPIPOX | A tool for Predicting Antigenic Peptides | http://imed.med.ucm.es/Tools/antigenic.pl | [77] |
| PEP-FOLD3 | A web-server for predicting peptide structures from amino acid sequences | https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/ | [78] |
| HeliQuest | A tool for calculation of helix properties | https://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py | [79] |

Eventually, new peptides would be generated by the *de novo* method based on amino acid patterns or frequencies. The discovery of sequence patterns, critical residue locations, and amino acid frequencies from eminent antimicrobial peptides serves as the basis for this strategy. As a result, this data is utilized to create prediction models and linguistic models to find novel AMPs [48, 49]. Houyvet et al. used the *de novo* method to design peptides using a pipeline. The free database ADP was used in the first step. Length, charge, hydrophobic percentage, possible structure, and pattern association were the criteria they used. They chose eight sequences as final candidates and tested them using four different online prediction algorithms accessible on the CAMPR3 database website to estimate the antibacterial potential of selected peptides. The findings revealed that two of the eight peptides tested had a broad range of antibacterial activity but did not cause hemolysis. Further, for some strains, both MICs and MBCs were minimal, making them more selective for these bacteria. These findings suggested that de novo peptide design could be a viable alternative to traditional peptide purification procedures, which need animal tissues and are time-consuming [50].

On the other hand, non-rational methods are based on the microbial surface display technology to obtain different well-established random peptide sequences for further screening depending on the current approaches in the peptide-based drug discovery process framework [6].

## Database resources dedicated to AMPs

The development and use of genomic and proteomic technologies have hastened the identification of AMPs. To properly handle AMPs, specific databases have been built. The peptide scope, style, kind of information, annotation, peptide search capacity, and prediction technique of these resources varies [51]. A list of different database resources dedicated to AMPs is represented in Table 1.

Furthermore, some online web servers are accessible to predict different characteristics of peptides or proteins such as anti-inflammatory, anti-bio-film, anti-microbial, and cell-penetrating activities. Acquaintance with them is indispensable to design novel peptides with less hemolytic and cytotoxic strength for human cells. For this reason, in Table 2, a list of useful online tools is provided.

The *de novo* approach is a method that has developed with the advent of various evaluation databases and software. However, the most problematic issue for novice researchers is generally how to use these online web servers. Whereas all web servers allow individuals to forecast the AMP quality of numerous peptide sequences at once, some have differing limits on the number of sequences that can be predicted. For example, the limit on the maximum number for ADP3 is one, for AMPGram is 50 and for MLAMP is 5. For Some servers such as $CAMP_{R3}$, DBAASP, ClassAMP users should upload sequences in the FASTA format.

These servers apply different prediction algorithms for antimicrobial peptides. For instance, the main algorithms for CAMPR3 are Support Vector Machines (SVM), Artificial Neural Network (ANN), Random Forests (RF), and Discriminant Analysis (DA). Users can choose one or all four algorithms for prediction. The users has three options on this online web server: scan the entire protein to predict its antimicrobial activity. Scan putative sequences to predict antimicrobial regions within proteins, and finally design rationally antimicrobial peptides by generating all possible single residue mutations and select the sequences having the highest AMP probability. The result is given a likelihood score (0 to 1) by RF, SVM, and ANN. The greater the likelihood, the more likely the peptide is antimicrobial [52]. Another example is DBAASPv3.0, which offers a prediction tool that may be used solely to predict linear peptides' antimicrobial activity. It is based on charge density, the hydrophobic moment and depth-dependent potential of peptides, among other physicochemical properties [54].

ADP3, the largest database of peptides, is one of the best references for researchers. It contains 3324 antimicrobial peptides with different activities, including antibacterial, antibiofilm, anti-MRSA, anti-TB, anti-endotoxin, anti-toxin, antiviral, anti-HIV, antifungal, anti-candida, anti-parasitic, Etc. This database has an antimicrobial peptide calculator and predictor. The user can acquire useful statistics such as peptide molecular weight, the proportion of each amino acid in the sequence, load, total hydrophobic ratio, and Bowman index by submitting a specific sequence. It is also possible to compare the sequence to all the sequences in the database and discover the most similar one to the given sequence.

Different modules are available on specialized web servers, such as dPABBs and HemoPI, to examine both peptides and protein fragments for specific activities such as antibiofilm or hemolytic potency. Since hemolysis of red blood cells by antimicrobial peptides is one of their weaknesses, the Hemolytic Potency module in HemoPI can be used to predict peptide hemolytic activity. It is based on two alternative prediction algorithms, SVM and SVM + motif, that users can choose from depending on their needs. Furthermore, this module creates all potential mutants of a particular peptide and forecasts each mutant's hemolytic activity as well as some relevant physicochemical attributes (like hydrophobicity, hydropathicity, charge, molecular weight and pI). While the protein mapping module is useful to predict hemotoxic regions in a protein, It can help users avoid selecting these regions for designing antimicrobial peptides.

As previously stated, the peptide's three-dimensional structure is critical to its performance. Determining the

three-dimensional structure of the peptide is a key characteristic that researchers should address while creating using the de novo method. The PEP-FOLD site is the best option for this, as it predicts a peptide's three-dimensional structure in minutes and offers it to the user after receiving a peptide sequence in FASTA format.

One of the issues with most of these servers is that the results are only available online, with no option to download the findings and review the data later. A limited number of servers like BIPEP send the prediction results to the user's email address.

## Structural modifications through the bio/chemical synthesis of new designed peptide

There are a number of approaches for synthesizing newly designed antimicrobial peptides. Fmoc-based solid-phase peptide synthesis and recombinant production methods are the most widely used. Today, Fmoc solid-phase peptide synthesis (SPPS) is the selected technique for peptide synthesis. High-quality Fmoc building blocks commercially are available at a reasonable price producing therapeutic peptides at low cost and on a grand scale. Many Fmoc building blocks produced from modified derivatives are commercially available resulting in synthetic access to various peptide derivatives straightforward. Although publications often suggest peptides with around 50-residue sequences as the average target that can be routinely synthesized, this condition practically is incomprehensible because many much shorter sequences are incredibly problematic, and the success of the synthesized peptide is not guaranteed (due to short half-life) [80].

On the other hand, AMPs can be recombinantly produced. *Escherichia coli* is the primary system used to produce recombinant AMPs because it can be quickly cultured, and post-translational modifications are not required for the activity of antimicrobial peptides as well. Despite these benefits, the direct expression of antimicrobial peptides in *Escherichia coli* is complicated. First, the antimicrobial property gives them a fatal feature towards the producing host. Secondly, they are highly susceptible to degradation by the proteolytic enzymes of the producer due to their small size and high cationic content. To overcome these limitations, several methods for producing recombinant AMPs coupled to a carrier that stabilizes the peptide have been reported. Small ubiquitin-like modifier (SUMO), biotin carboxyl carrier protein (BCCP), thioredoxin (Trx), green fluorescent protein (GFP), calmodulin, glutathione S-transferase (GST), and human serum albumin are some instances of carriers for the fusion peptides [81, 82].

The utilization of acidic partners is another approach for effectively producing antimicrobial peptides [83]. In all of these circumstances, the carriers or partners help the cells cope with the AMP's toxicity while enhancing their protein expression efficiencies. To retrieve the offered AMP, however, it is frequently required to eliminate these carriers, which necessitates the use of costly enzymatic cleavage or toxic chemicals. An alternate technique is to make antimicrobial peptides as protein nanoclusters or inclusion bodies. Inclusion bodies are porous, non-enveloped, and mechanically stable nanoparticles primarily shaped by a polypeptide and produced within the recombinant protein manufacturing process, with the potential to be a protein-slow release system when administered. Another benefit of this technology is that it produces proteins in a single step rather than requiring the manufacture of carriers and the separation of a biomolecule, as in other ways [84]. Different strategies to produce recombinant AMPs are shown in Fig. 3.

## *In vitro* antimicrobial activity assays

To use antimicrobial peptides as therapeutic agents assessing their antimicrobial activity before clinical applications is fundamental. One of the most substantial assays is to evaluate the minimum inhibitory concentration (MIC) of antimicrobial peptides, which is the lowest concentration of antimicrobial agent able to inhibit the microorganisms' growth visibly. To calculate MIC, macro- and microdilution procedures are considered quantitative. The sensitivity, repeatability, and simplicity of having commercial plates prepared with antibiotics, the economy of space and chemicals, and the ability to employ automated reading devices to expedite the preparation of reports are all advantages of adopting micro-dilution. Because of these benefits, this

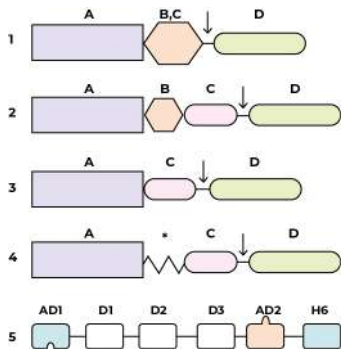


**Fig. 3** A schematic representation of fusion proteins for the recombinant production of AMPs. Sections 1 to 4 represent different ways to link carrier to AMP. Section 5 shows a schematic representation of AMPs to produce inclusion body nanoparticles. A: fusion tag1; B: fusion tag2; C: cleavage site; D: antimicrobial peptide; *: spacer; AD1 and AD2: aggregation-seeding domains; D1, D2, and D3: different domains of the desired peptide; H6: histidine tag. In Sect. 5, dashes between the domains represent linker sequences

approach is now widely used to determine the MIC of drugs with potential antibacterial activity [85]. A schematic scheme of micro-dilution test are shown in Fig. 4.

Considering the resistance of biofilm bacteria to commercial antibiotics, evaluating the capacity of AMPs to inhibit biofilm formation is becoming increasingly important. A microtiter dish assay is a valuable tool for the study of the early stages in biofilm formation [86]. In addition, to verify the safety of the potential clinical application of AMPs as antibiotic candidates with various bio-activities, performing a hemolysis test is essential [61]. Cellular wound healing activity, anti-viral and anti-inflammatory activities, cell proliferation assays, and evaluation of stability against bacterial protease and human intestinal enzymes conduct as other commonly used techniques before entering their clinical phases [87]. Structural studies by circular dichroism to assess conformational changes in different buffer conditions

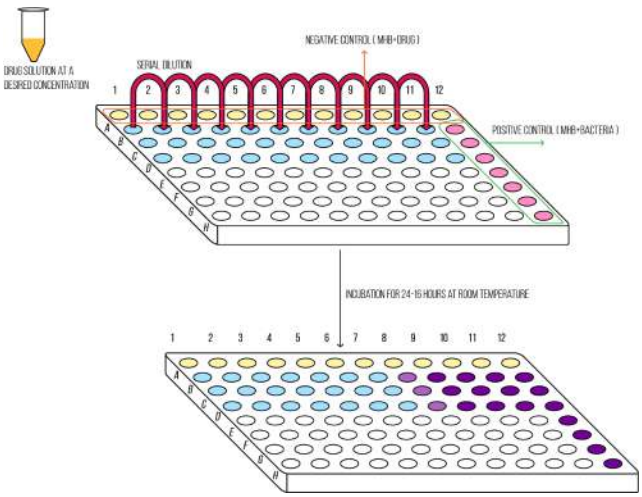


**Fig. 4** A schematic representation of the microdilution method used for antimicrobial activity assay. In this method, 96-well microplates are used. Negative control includes the medium and the desired peptide, and positive control includes the culture medium and bacteria. Pour a specific volume of peptide in the first well and reduce the dilution of the subsequent wells by pipetting so that the positive control well will be free of the peptide. Then add a particular concentration of bacteria to each well and incubate for 16 to 24 h at room temperature. Finally, the bacterial growth rate is assessed in each well. A well where bacteria have not grown indicates a concentration of peptide that inhibits bacterial growth. Yellow wells: indicate negative controls containing the peptide and medium (usually Mueller Hinton Broth culture medium); pink wells: show positive controls having medium and bacteria; blue wells: indicate the combination of culture medium, bacteria, and the peptide solution with different concentrations. The dark purple color indicates that bacteria have grown. Positive control turned dark purple after 16 h, indicating that the bacteria had grown well. Bright purple indicates that the peptide has somewhat reduced bacterial growth in the well, but its concentration was insufficient to inhibit growth completely. The blue wells in the second micro-plate indicate that the peptide had inhibited bacterial growth, and the lowest concentration that inhibited bacterial growth is reported as the minimum inhibitory concentration

is also recommend because it helps find the best environment in which a desired antimicrobial peptide is stable with the most efficiency [88].

## AMPs as wound healing agents

Many AMPs enhance wound healing and increase angiogenesis as essential components in the tissue regeneration process, in addition to their antibacterial action. For instance, research was shown that human β-Defensins, Cathelicidin LL-37, and Dermicidins, which are expressed on the skin, promote wound-healing besides their antibacterial activity; various studies have investigated their use in dermal lesions dressings, especially diabetic foot ulcer (DUF) [89]. LL-37 was utilized to treat Diabetic Foot Ulcer (DFU) and has been proven to improve dermal cell angiogenesis, migration, and proliferation as a critical factor in wound healing in phase II clinical studies [90].

In non-diabetic and non-infectious mice and porcine specimens afflicted with *Staphylococcus aureus*, IDR-1018, an intrinsic defense-regulating peptide with less cytotoxicity than LL-37, was demonstrated to speed significantly wound healing [90]. In pig specimens bearing diabetic wounds infected by Staphylococcus aureus, human beta-defensin (hBD-2) stimulates migration of keratinocytes, whereas hBD-3 has been shown to speed wound closure by decreasing bacteria ten times [91]. In addition, hBD-3 and LL-37 were discovered to activate skin cells and corneal epithelial cells, resulting in improved ocular surface healing. LL-37 causes respiratory system cells to migrate, proliferate, and close wounds [92].

Several performed studies on human saliva factors have reported that Histatin 1 [93], Histatin 2, Histatin 3 [94], which are members of the prominent family of antimicrobial peptides in saliva, play roles as the significant repair factors in the wound healing in saliva. Another study has demonstrated that Histatin 5, a proteolytic product of Histatin 3, also has a powerful wound-healing effect by increasing epithelial migration in various human cells [95]. Histatins' two actions, namely antibacterial properties and cellular stimulants, are incredibly different in function, structural necessities, and specificity. Antimicrobial action is concerned with damaging the target cell's phospholipid membrane, which is reliant on the peptide's chirality. Its stimulating ability in host cells, on the other hand, involves a stereospecific interaction with a hypothesized membrane receptor. Oudhoff et al. showed by stepwise-truncation that the SHREFPFYGDYGS domain of the sequence of parental Hst-1 38-mer is essential for this peptide activity.

Further, cyclization through binding the N-terminal to the C-terminal of Hst-1 increases its activity up to 1000 fold

[96]. Pixiganan, frequently referred to as MSI-78, is also another AMP with a well-established therapeutic potential for DFU. It is a native AMP extracted from the skin of an African toad, *Xenopus laevis*, and is thought to be a Magainins homolog. Early in clinical studies, Pexiganan was demonstrated to promote dermal cell migration and antibacterial efficacy against both Gram-positive and Gram-negative bacteria in diabetic foot ulcer species. Locilex, a 0.8% Pixiganan cream, was subsequently developed and patented to treat diabetic foot ulcers. Locilex was eventually shown to be ineffective in clinical phase III studies compared to a carrier cream without Pixiganan [89].

Magnesians and related peptides are not the only wound-healing peptides secreted from amphibian skin, like frogs, caecilians, toads, and salamanders. In fact, not only numerous of the well-known peptides have an amphibian, but they also exhibit wound healing properties [89]. CW49 seems to be a small peptide with just eleven residues that its origin is the skin of the frog *Odorrana graham*. It promotes angiogenesis and has been demonstrated to avoid an excessive inflammatory response when tested in DFU. [97, 98]. There is also an extensive collection of other AMPs of diverse origins that show the ability to heal chronic wounds, especially DFU. For example, Temporins A and B are attractive candidates as new therapeutic agents for skin infections and soft tissues with *S. aureus* [99].

The instability of AMPs is a significant problem because even in a saline solution, most AMPs can self-assemble and lose their function. They may also be susceptible to enzymatic breakdown [7]. Nishikawa et al., for example, developed AG-30, an AMP with α-helical structure which demonstrates angiogenesis properties in a mouse ischemic limb specimen. Using the membrane degradation process, AG-30 has high antibacterial action against *P. aeruginosa, E. coli, and S. aureus*; however, it is unsustainable in saline solution [100]. The cationic AG-30/5 C was created by replacing five AG-30 residues with five cationic amino acids, resulting in a more stable form of AG-30. Consequently, it has demonstrated respectable saline solution sustainability for at least 12 months at 5 ° C. In vitro, AG-30/5 C displayed potent antibacterial action *against P. aeruginosa, S. aureus, Candida*, and MRSA besides regenerating epithelial cells and remarkably promoting angiogenesis in MRSA-infected mice and porcine models. Compared to the LL-37-healed control group, it also considerably enhanced blood flow surrounding the wound [101].

Another cationic peptide from frogs' skin causes wound closure by stimulating transforming growth factor β-1 (TGFβ-1) in full-thickness mice models. Tylotoin enhances angiogenesis by increasing epithelial cell tube formation and speeds wound healing by stimulating TGF-1 production and IL-6, both involved in wound healing. It has been demonstrated that increased cell mobility and proliferation of keratinocytes and fibroblasts in vitro and granulated tissue development occur in the damaged region of the full-thickness mouse model [102]. A small peptide called Triger 17 leads to wound healing *in vitro* and *in vivo*. This peptide accelerates wound closure in a mouse full-thickness wound model by speeding up keratinocyte migration and proliferation. It causes macrophage activation in the inflammation site by causing keratinocytes to migrate and proliferate. Moreover, Triger17 stimulates releasing IL-6, TGF-β1, and TGF-a, all of which play a crucial role in wound regeneration [103]. Another small peptide, ERL3 (13-mer), has strong cell selectivity and can accurately eliminate MRSA cells produced in a co-culture model by membrane rupture while also successfully destroying MRSA biofilms. In a burned mouse infected with MRSA, WRL3 effectively kills germs *in vivo* by increasing cytokine release, attracting macrophages, and encouraging angiogenesis and wound healing. It was also shown to be more effective than vancomycin at preventing MRSA infections. Consequently, WRL3 might be utilized to treat infections associated with MSRA in a burned skin lesion and promote wound healing [104]. Epinecidin-1 (Epi-1) is another AMP that has been patented as a stimulus of proliferation and migration of keratinocytes *in vitro*. It also showed complete healing in heat-burnt pig skin infected with MRSA after Twenty-five days. Epi-1 increases angiogenesis, epithelial function, and collagen production around injured sites, according to the findings [105].

Recently, some AMPs were unsuccessful in the clinical trials. Pixiganan, as an example, failed over the clinical phase III while being used to treat infections in DFU. As an analog of Protegrin-1, Iseganan had been made to prevent multi-microbial infections in the mouth, but it failed in the clinical phase III as well [106]. These failures have been attributed to significant ineffectiveness compared to other antibacterial drugs or several side effects [8].

## Conclusion

Antimicrobial peptides are crucial not only in overcoming microbial resistance and fighting bio-films but also as agents for the treatment of various diseases due to their multi-functionality. Because of their short half-life in physiological pH, diminished activity under situations of extreme salt, divalent cations, and variable pH, straightforward digestion through proteinases, and finally toxicity against eukaryotic cells, most peptides have been used topically to treat chronic wound infections to date. Using them topically to treat wounds has the benefits of (a) direct antimicrobial activity that prevents infection or otherwise wound healing

would be delayed, (b) binding and inactivating compounds such as lipopolysaccharides that reduce pro-inflammatory damages, and (c) some direct effects on cellular behaviors such as enhanced migration and proliferation.

Since the extraction of natural peptides from various organisms is challenging due to lack of resources and a time-consuming process, researchers have developed new methods for designing and synthesizing peptides. Logical and irrational design are two ways to find new peptides. To this end, databases dedicated to various antimicrobial peptides have been created, and valuable online tools have been developed. However, users have been puzzled by the existence of multiple servers and databases. A crucial question that cannot be answered is which server or online tool is more accurate. Nevertheless, one option is to use several servers to compare their results and select common sequences. Because separate servers employ different algorithms for prediction, the accuracy of the results can be improved, and a sequence classified as an antimicrobial peptide by many servers is more likely to have stronger antibacterial capabilities. Additionally, experimental testing must be done to corroborate the results of the computational approaches before the identified peptides can be introduced as antimicrobial peptides. For this purpose, various tests have been designed to evaluate the performance of the designed peptides to obtain the most efficient peptide. There is also great hope for using these peptides to treat various viral diseases, cancers, parasites and even regulate immune pathways.

## Declarations

**Conflict of interest** The authors declared that there is no conflict of interest.

**Compliance with Ethical Standards** This article does not contain any studies with animals performed by any of the authors and also, does not contain any studies with human participants performed by any of the authors.

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