Review

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Short native α-helical cationic antimicrobial peptides: promising alternative antibiotics

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Abstract

An emergence increasing evidence of antibiotic resistance in microorganisms has resulted in reduced effectiveness of available conventional antimicrobial drugs and becomes a major concern for public health worldwide. Antimicrobial peptides (AMPs) are regarded as excellent candidates of promising alternative antibiotics owing to their higher or equal potencies and broad spectrum antimicrobial activities with less possibility of resistance induction when compared to conventional antibiotic drugs. Among AMPs, short native α-helical cationic antimicrobial peptides have been illustrated as potential antibiotic candidates because they contain small and simple structure providing the advantages for chemical modifications and structure-activity relationship studies. Interestingly, the small and simple structures are attractive and demonstrate as commercially feasible candidates for further development in therapeutic or industrial uses. Therefore, this review focused on current discovered short native α-helical cationic antimicrobial peptides (≤ 10 amino acid residues in length) which are anoplin, temporin-H, and temporin-SHf. This review also summarized these peptides in aspect of physico-chemical properties, activities and toxicities.

Keywords: Antimicrobial peptide, Cationic peptide, Antibiotics, Anoplin, Temporin-H, Temporin-SHf

Introduction

An increasing occurrence of antibiotic resistance in human-pathogenic microorganisms has resulted in reduced effectiveness of available conventional antibiotics and becomes a major concern for public health [1]. Novel promising antimicrobial agents have been discovered, studied and developed to overcome this problem. Among them, antimicrobial peptides (AMPs) are regarded as excellent candidates owing to their higher or equal potencies and broad spectrum antimicrobial activities with less possibility of resistance induction when compared to conventional antibiotic drugs [2]. Antimicrobial peptides (AMPs) are natural peptides presented in microorganisms, plants, and also in animals playing a critical role in innate non-specific host defense system. AMPs confer resistance against infections without prior exposure to foreign pathogens. Therefore, AMPs sometimes are alternatively called "host defense peptides (HDPs)" due to their host-defense functions [3, 4]. AMPs are diverse in sequence, length, and structure. However, they can be classified

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into four major classes based on their secondary structures which are alpha-helical peptides, beta-sheet peptides with disulfide bridges formed by cysteine residues, extended structure enriched in certain residues i.e. arginine, tryptophan, and peptide containing mixed structure [5]. Although their secondary structures are diverse, AMPs share common features such as low molecular weight, net positive charge, and amphipathicity. These features are believed to govern activities of AMPs [6]. There are several modes of actions of AMPs in order to against microorganisms; however, the mechanism involved in membrane perturbation has believed to be a major mechanism. Therefore, AMPs have been classified as a class of "membrane-active peptides" due to their major mode of action [7].

AMPs share a common three-dimensional arrangement, as they fold into amphiphilic molecules that have charged and hydrophilic portions segregated from the hydrophobic portion. This amphiphilic organization allows for strong interactions between the positively charged peptides and negatively charged bacterial membranes, whilst the hydrophobic groups facilitate the penetration of the AMPs into the lipid phase of the membrane [7, 8]. Many AMPs are proved to exhibit notable broad spectrum antimicrobial activities against susceptible human-pathogenic microorganisms. Interestingly, many AMPs appear to selectively exert antimicrobial activity in prokaryotic cells, but they demonstrate no cytotoxicity in normal eukaryotic cells [4]. AMPs have been reported to act via different mechanisms involving membrane destabilization and disintegration of target cell membranes unlike the conventional antibiotics [9].

More importantly, the major mechanism of AMPs involved with membrane destabilization and disintegration of target cell membranes, unlike the receptor-based mechanism of conventional antibiotics, suggesting a less likelihood to induce antibiotic resistance [2, 3, 9]. These advantages consequently enable AMPs to become a spotlight for extensive pharmaceutical research with attempts to develop novel anti-infective drugs base on AMPs and to cope with a persisting problem of microbial resistance in conventional antibiotics. However, the costs of peptide drug development and production for pharmaceutical industry are usually more expensive than that of conventional antibiotics. Therefore, many research groups have attempted to explore the novel potential short native AMPs to be used as antibiotic drug candidates since the shorter peptides are less expensive in production cost than that of the longer peptides containing the same activity. The aims of this review are to explore the current discovered short native α-helical cationic antimicrobial peptides ($\leq$ 10 amino acid residues in length) by retrieving the lists of the peptide via "the Antimicrobial Peptide Database (APD)" [10]. By data extracting for this database, there are only three short native α-helical cationic antimicrobial peptides currently available which are anoplin, temporin-H, and temporin-SHf. Interestingly, two short native AMPs, anoplin and temporin-SHf, demonstrate high potency and low toxicity that meet the requirement to be promising novel antibiotic candidates.

Table 1 Examples of antimicrobial peptides and their mode of activities

Mode of antimicrobial activity	Examples of peptides
Transmembrane pore-forming mechanisms	
Toroidal pore (Wormhole, disk)	LL-37 [11]
Carpet	Dermaseptin S [12]
Barrel stave (Helical-bundle model)	Alamethicin [13]
Modes of intracellular killing	
Alters cytoplasmic membrane septum formation	Indolicidin [14]
Inhibits cell-wall synthesis	Mersacidin [15]
Binds nucleic acids	Buforin II [16]
Inhibits DNA/RNA and protein synthesis	Pleurocidin [17]
Inhibits enzymatic activity	Histatins,[18]

Mode of antimicrobial actions

Despite the sequence and structural diversities, AMPs do share common characteristics that are believed to govern their activities. AMPs are almost low molecular weight peptides composed of less than 50 amino acid residues and often contain high proportion of cationic (basic) amino acids i.e. arginine (Arg) or lysine (Lys). Most alpha-helical AMPs usually possess amphipathicity since they have been found hydrophilic residues including basic amino acids together with hydrophobic residues i.e. phenylalanine (Phe) or tryptophan (Trp) [2, 7]. Interestingly, net positively charge and amphipathicity of AMPs are considered to be crucial for the antimicrobial activities. Cationic residues electrostatically facilitate an initial interaction with negatively charged target membrane while hydrophobic residues enable the peptides to partition into the membrane lipid bilayer [7, 8].

Most AMPs are membrane active peptides and their mechanisms involve with membrane permeabilization of target microorganisms. However, other mechanisms have also been reported such as intracellular targeting (Table 1). Both membrane perturbation and intracellular inhibiting also required the peptide-membrane interaction to be an initial necessary step [9].

The selectivity of AMPs is believed to largely depend on the differences in the membrane structures of target microorganisms and the membrane structures of hosts. The compositions of mammalian cell membranes clearly differ from bacterial membranes. The outermost leaflet of the bilayer of most bacterial membranes predominantly constitutes lipids with zwitterionic phosphatidylethanolamine (PE), anionic phosphatidylglycerol (PG), and cardiolipin (CL), giving an overall negatively charged surface [19]. Thus, a preferential binding between cationic AMPs and anionic phospholipids occurs by electrostatic interaction which is relatively strong. Not only acidic phospholipids, bacterial membranes also contain negatively charged lipopolysaccharides (in Gram-negative bacteria) or teichoic acid (in Gram-positive bacteria) which could be the other key factors for preferential binding of cationic AMPs. For eukaryotic cells, zwitterionic phosphatidylcholine (PC) and sphingomyelin (SM) are mainly constituted in the outer leaflet as opposed to the membrane of prokaryotic cells whereas PE and anionic phosphatidylserine (PS) are mostly confined in the inner leaflet, conferring no net charge [20]. Therefore, hydrophobic interaction occurred between the hydrophobic face of AMPs and zwitterionic phospholipids

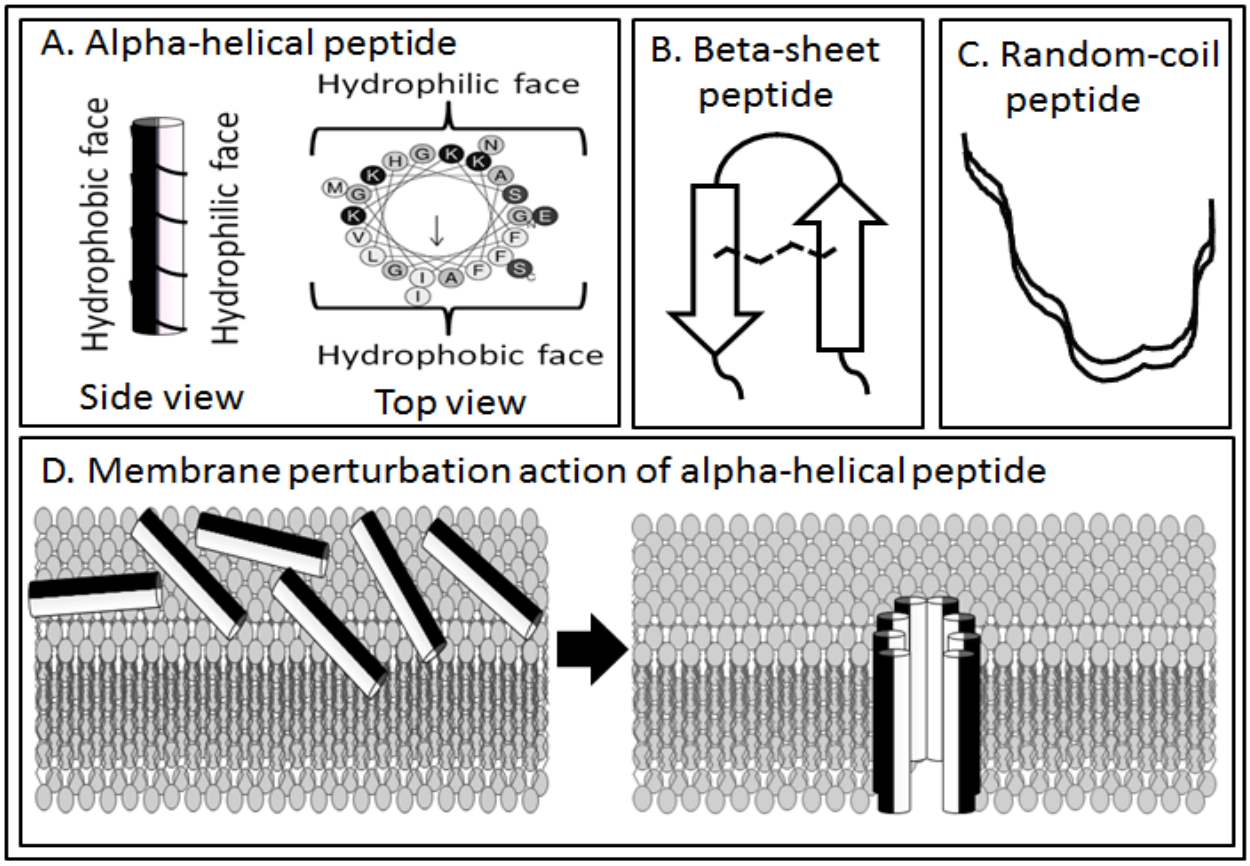

Figure 1 The secondary amphiphilicity of α-helical antimicrobial peptide. The α-helical antimicrobial peptide (A) possesses secondary amphiphilicity and this architecture is more expedient for peptide design comparing to beta-sheet (B) and random-coil structures (C). Predicted model for membrane perturbation action of α-helical antimicrobial peptide (D) has been proved.

Table 2 Physicochemical properties of Anoplin, Temporin-H, and Temporin-SHf

Physicochemical properties	Short native α-helical cationic antimicrobial peptides		
	Anoplin	**Temporin-H**	**Temporin-SHf**
Length	10 residues	10 residues	8 residues
Molecular weight (MW)	1153.51 Da	1096.37 Da	1075.31 Da
Isoelectric point (pI)	11.82	9.69	10.55
Net charge at physiological pH (7.4)	+4	+2	+2
Hydrophobic residues (%)	50%	50%	75%
Boman Index	0.3 kcal/mol	-0.56 kcal/mol	-0.42 kcal/mol

on the outer membrane surface; however, such interaction is weaker when compared to the electrostatic interaction. Therefore, AMPs demonstrate the preferential binding to the membrane of pathogenic microorganisms rather than the membrane of mammalian cell [4].

In conclusion, AMPs must interact with the target membranes as part of their direct antibacterial mechanisms of action, resulting in membrane perturbation and disruption together with destroying membrane-associated physiological events such as cell wall biosynthesis or cell division, and/or translocation across the membrane to interact with cytoplasmic targets. The peptide is then inserted and altered membrane structure, including thinning, pore formation, altered curvature. After translocation, the peptides diffuse into the cytoplasm to reach intracellular targets. These basic mechanisms usually explain many aspects of the observed antibacterial activity of AMPs (Table 1) [8,9].

α-Helical Cationic AMPS

Among classes of AMPs, α-helical cationic AMPs are one of the groups that have been widely studied for structure-activity relationship. Comparing to random-coil and beta-sheet structure, α-helical peptide architecture is more expedient for peptide design and development and structure-activity relationship studies. The α-helical antimicrobial peptides possess secondary amphiphilicity i.e. a spatial segregation of hydrophobic and hydrophilic residues about the α-helical long axis (Figure 1) [9] as shown by helical wheel projection of LL37 and magainin 2 analysed by Heliquest programme [21] (Figure 2). The α-helical AMPs generally require the presence of an amphiphilic interface which allows the non-polar face of their α-helical structures to interact with the membrane lipid core while concomitantly permitting its hydrophilic face to engage in electrostatic interactions with the membrane lipid polar head group region (Figure 1). Magainin 2 is a well characterized α-helical cationic AMP composed of 23 residues isolated from the skin of the African clawed frog. NMR studies showed that magainin 2 form amphipathic helical structures in 25% trifluoroethanol [22]. Magainin 2 conserved strong secondary amphiphilicity as shown by helical wheel projection (Figure 2). One side of α-helix of magainin 2 illustrates polar amino acids (polar face of peptide), but the other side of α-helix of magainin 2 presents nonpolar residues (nonpolar face of peptide). This amphiphilicity demonstrated to be a key factor involving in antimicrobial function.

According to the mode of membrane perturbation action, α-helical cationic AMPs interact with membrane surface and increase the permeability of cell membrane which is an ultimate step that leads to cell death. The three following models have been proposed which are the carpet model, the toroidal pore model, and the barrel-stave model. For the carpet model, AMPs must align parallel to the surface in a carpet-like manner and fully cover at the surface of target cell membrane of microorganisms and then the membrane starts to disintegrate and sink in a detergent-like fashion resulting in a rapid cell lysis. For the toroidal pore model, AMPs must insert into the membrane and induce the lipid to bend and generate pores. The peptide-membrane interaction demonstrated the pattern that the inserted peptide and the lipid head groups are lining along the water core. For the barrel-stave model, α-helical AMPs insert perpendicularly into the membrane bilayer in a barrel-like bundles forming a transmembrane pore where hydrophobic region of the peptides align with the lipid core region and the hydrophilic surface facing the interior region of the pore [23].

Promising short AMPS for alternative antibiotics

As previously mentioned, the cost of peptide drug production for pharmaceutical industry is usually more

expensive than that of conventional antibiotics. Thus, many researches have focused on investigating the novel potential short native AMPs to be developed as novel antibiotics since the shorter peptides are less expensive for production cost than that of the longer peptides containing the same activity. The list of short native α-helical cationic antimicrobial peptides was retrieved from the Antimicrobial Peptide Database (APD) [10]. This database currently contains 2411 antimicrobial peptides (236 bacteriocins, 6 protozoan antibacterial peptides, 12 fungal AMPs, 308 plant AMPs, and 1805 animal host defense peptides). In addition, this database also classified all peptides by their activities including antibacterial peptides, antiviral peptides, antifungal peptides, antiparasitic peptides, anticancer/tumor peptides, anti-protist peptides, insecticidal peptides, spermicidal peptides, anti-HIV-1 peptides, and AMPs with chemotactic activity. In order to retrieve the list of short native α-helical cationic antimicrobial peptides, three inclusion criteria were set up. Firstly, the peptides must be α-helical structure. Secondly, the peptides must be cationic peptides. Finally, the peptides must be decapeptides or shorter in length. The non-native peptides, modified peptides, and synthetic peptides must be excluded from this review. Three short native α-helical cationic antimicrobial peptides were extracted from the APD which are anoplin, temporin-H, and temporin-SHf (Figure 3). The physicochemical proterties of these peptides retrieved from the APD are illustrated in Table 2.

Anoplin: Anoplin was isolated and purified from the venom sac of female solitary wasp *Anoplius samariensis*. It composed of ten amino acid residues with C-terminus amidation, (GLLKRILTLL-NH_2) [24]. The molecular weight of anoplin is 1153.51 daltons. The percentage of hydrophobic amino acid of anoplin is similar to that of temporin-H which is 50%. This peptide can dissolve in water and aqueous buffer. Moreover, the peptide demonstrated as amphipathic molecule containing net charge +4 with isoelectric point value of 11.82. With the Boman index value of 0.30 kcal/mol, this low value indicated that the anoplin can be a good antibacterial drug candidate without many side-effects because it shows low potential for interaction with other proteins and receptors (Table 2).

The secondary structure of anoplin illustrated the helical conformation determined by circular dichroism (CD) spectroscopy. The CD spectra of anoplin illustrated random-coil characters in Tris buffer and helical characters in amphipathic environments such as in TFE/water mixture and SDS solution [25]. The peptide sequence of anoplin also clearly presented amphipathic helical characteristic when predicted with helical wheel projection [26]. For membrane perturbation analysis, anoplin was investigated its ability by leakage experiments. In this test, carboxyfluorescein dye was trapped in various membrane models including zwitterionic membrane model and anionic membrane model. The results indicated that anoplin can induce membrane disruption and it has ability to induce the formation of channel- or pore-like structure in anionic membrane model at the same concentration at which the antimicrobial activity has been observed [25].

For antimicrobial activities, this decapeptide presented broad spectrum antimicrobial activity against both Gram-positive (*S. aureus, S. saprophyticus*, and *B. subtilis*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*) with the MICs values in the range of 5-50 μg/ml. Even though, the presence of high salt condition

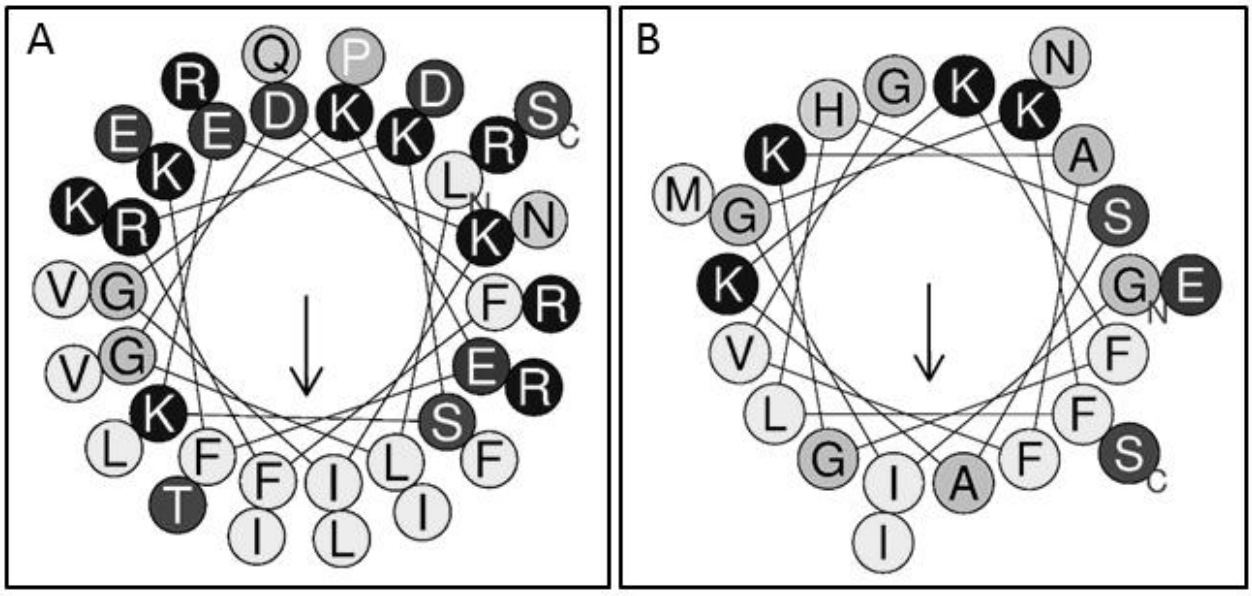

Figure 2 Helical wheel projections of (A) LL37 and (B) Magainin 2. Helical wheel projections of LL37 and Magainin 2 were analyzed by Heliquest programme. The arrow is a vector indicating the direction the hydrophobic moment, pointing towards the hydrophobic face of peptides.

affected this activity [24]. For antibacterial activities against resistance strains, anoplin has also been tested in order to investigate its antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE). The data indicated that it can inhibit these resistant strains with the MICs of 512.0 μM and 132.7 μM, respectively [27]. For antifungal activity, anoplin demonstrated antifungal activity against *Leptosphaeria maculans* and *Brassica napus*. In addition, the data also illustrated that the amidation at carboxy-terminus is not necessary for antifungal activity [28]. For cytotoxic analysis, anoplin displayed relatively low hemolytic activity in human erythrocytes [24].

The structure activity relationship (SAR) of anoplin has been observed [26, 29]. Anoplin and its analogues have been created for the SAR observations. Alanine-positional scanning, C- and N-terminus truncations and amino acid substitution studies were performed and the relationships were interpreted from several values such as minimum inhibitory concentration (MIC), peptide concentration causing 50 % hemolysis (EC_{50}), mean hydrophobicity, mean hydrophobic moment, retention time of RP-HPLC and the percentage of helicity. The truncated analogues indicated that the full length with ten residues must require remaining antimicrobial activity. The two potential anoplin analogues were found out from alanine-positional scanning, ano-A5 and ano-A8, based on the improved antibacterial activity with low hemolytic activity [26, 29]. The membrane permeabilization of anoplin was determined by cell membrane vesicle leakage experiments indicated that anoplin enabled to interact with cell membrane and caused cell membrane leakage. This process required electrostatic interaction between anoplin and membranes that depend on positively net charge of anoplin and negatively net charge from lipid composition of membranes [25].

Temporin-H: The temporin family includes more than 40 members isolated from secretions of European red frog, *Rana temporaria* [30]. Temporins are all amidated at the C-terminus; those containing one basic amino acid residue, either lysine or arginine, in the peptide sequence. Temporins illustrated their antibacterial activities against Gram-positive bacteria and *Candida albicans*. The properties of peptides extracted from the European red frog and will be called temporins should contain the proterties which include the following: (a) amphipathic α-helical AMPs (10–14 amino acids); (b) the low net positive charge at a neutral pH, ranging from 0 to +3; (c) acting efficiently and rapidly against a wide range of pathogens (bacteria, viruses, fungi, yeasts and protozoa) without toxic to normal mammalian cells in some temporins; (d) perturbation of the cytoplasmic membrane for their mode of action; (e) displaying immunomodulatory effects in some temporins [31]. Temporins are mainly active on Gram-positive bacteria, including clinically isolated methicillin-resistant *Staphylococcus aureus (MRSA)* and vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* with minimal inhibitory concentrations ranging from 2.5 to 20 mM [32, 33, 34].

Temporin-H is one of the short native α-helical cationic antimicrobial peptides in temporin group.

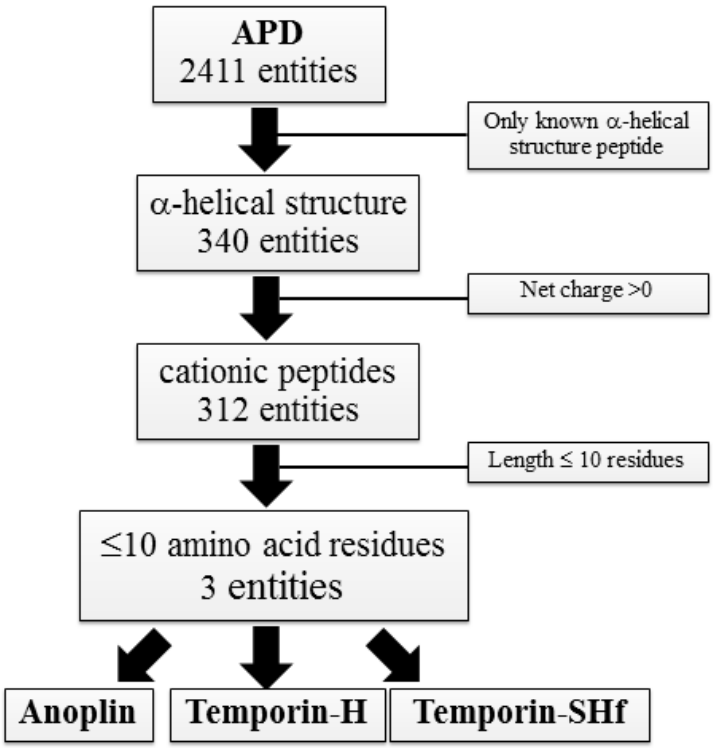

Figure 3 Short native α-helical cationic antimicrobial peptides retrieve from Antimicrobial Peptide Database (APD).

It contains only ten amino acid residues in length with amidated at the C-terminus (LSPNLLKSLL–NH_2). Temporin-H has molecular mass only 1096.37 daltons. The percentage of hydrophobic amino acid of temporin-H is equal to that of anoplin which is 50%. This peptide can use water or aqueous buffer as diluent. Similar to other AMPs, temporin-H demonstrated as amphipathic molecule containing net charge +2 with isoelectric point value of 9.69. The Boman index of this peptide is very low with the value of -0.56 kcal/mol indicating that the temporin-H can be a good antibacterial drug candidate without many side-effects because it shows low potential for interaction with other proteins or receptors (Table 2). The secondary structure of temporin-H was investigated by using circular dichroism (CD) spectropolarimeter. The CD spectra of temporin-H were recorded in water and after addition of trifluoroethanol. The data demonstrated that an increase in trifluoroethanol concentration caused a progressive change from a random conformation to an α-helical structure, the effect being complete at about 30% trifluoroethanol [32].

For membrane perturbation analysis of temporin-H, the peptide was tested by calcein leakage experiment. The eggyolk L-α-phosphatidylcholine (PtdCho) and bovine brain L-α-phosphatidyl-L-serine (PtdSer) liposomes were used to be membrane models represented of zwitterionic and anionic membrane models, respectively. The results indicated that temporin-H demonstrated the membrane perturbation activity. However, the abilities to induce calcein leakage of membranes are lower than those of temporin-A and temporin-B [32].

For antibacterial activities, temporin-H does not kill bacteria. The antibacterial activities of this peptide against *E. coli* D21 and *S. aureus* were illustrated that temporin H is completely inactive. However, temporin-H acts synergistically against Gram-negative strains when combined with classical antibiotics [31, 32]. The synergistically antibiotic activity was examined in *E. coli* D21. With rifampicin, temporin-H demonstrated the synergistically antibacterial function comparing to rifampicin alone and temporin-H without rifampicin. For cytotoxicity, the data illustrated no hemolytic activity of this peptide when examined with human erythrocytes [32].

Temporin-SHf: Temporin-SHf is a new type of short native α-helical cationic antimicrobial peptides. It was isolated and cloned from the skin of the frog *Pelophylax saharica*. It is an ultrashort with only eight residues in length (FFFLSRIF-NH_2) and also contains C-terminal amidation. The molecular mass of temporin-SHf is only 1075.31 daltons. The amino acid sequence of temporin-SHf contains highly hydrophobic sequence with the highest percentage of Phe residue of any known peptide or protein with 75% of hydrophobic residues. However, this peptide can be soluble in water and also in aqueous buffer. Moreover, the peptide demonstrated as amphipathic molecule containing net charge +2 with isoelectric point value of 10.55. The Boman index of this peptide is very low with the value of -0.42 kcal/mol indicating that the temporin-SHf can be a good antibacterial drug candidate without many side-effects because it shows low potential for interaction with other proteins and receptors (Table 2). For secondary structure, the peptide demonstrates a well-defined α-helical structure from residue 3 to 8 examining by CD and NMR spectroscopy combined with restrained molecular dynamics calculations, when bound to zwitterionic dodecyl phosphocholine or anionic SDS micelles [35]. For antimicrobial activities, temporin-SHf has broad-spectrum microbicidal activity against Gram-positive and Gram-negative bacteria and also yeasts. Interestingly, it demonstrated no hemolytic activity and this is one of the key properties indicating that temporin-SHf may be a proposing candidate of antibiotics. The mechanism of action of temporin-SHf was found to be membrane perturbation mechanism. Unlike longer AMPs, temporins-SHf is not long enough to form toroidal pores through the target membrane. Instead, results from DSC and NMR spectroscopy studies suggested that the peptide destabilizes anionic lipids through a detergent-like effect or via the carpet mechanism [36].

Temporin-SHf induced permeabilization of the bacterial cytoplasmic membrane. The study was examined by incubating *E. coli* ML-35p with the peptide (2–100 μM) and observed by measuring the time-dependent hydrolysis of the small chromogenic substrate ONPG into ONP by cytoplasmic galactosidase. The data indicated that membrane permeabilization/disruption of bacteria by temporin-SHf induced the leakage of the intracellular content and was concomitant with cell death [36]. The antibacterial activities of temporin-SHf were examined in various microorganisms. Temporin-SHf was highly active against *B. megaterium, S. aureus, E. coli*, and *S. cerevisiae* with MIC values in the range of 3–30 μM. The peptides illustrated bactericidal activity for *S. aureus* and *E. coli* since there is no visible colony presented when MIC well contents of these two bacteria were spread on agar plates. For activities against resistant strains such as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA) and vancomycin-resistant enterococci (VRE), there is no data available until now. For antifungal activity, temporin-SHf were examined its activity against filamentous fungus *A. flavus*. The data indicated that it is weakly active for filamentous fungus. Interestingly, temporin-SHf contains no cytotoxic against human erythrocytes (LC_{50}= 200 μM), with a hemolytic activity detected only at peptide concentration far above the MIC values determined for the sensitive strains (3–50 μM) [36].

Conclusions

According to the rapid emergence of antibiotic resistance in human pathogenic microorganisms of conventional antibiotics, α-helical cationic antimicrobial peptides have attracted considerable interest as a possible new generation of anti-infective candidates. However, low cost of drug development for therapeutic or industrial purpose requires small peptide with simple structure. Therefore, considerable research has been devoted to

optimizing peptide length combined with a simple design. Antimicrobial Peptide Database (APD) currently contains 2411 antimicrobial peptides which we can use this database to obtain the promise short native α-helical cationic AMPs. Data retrieving from this database illustrated only three short native α-helical cationic AMPs which are anoplin, temporin-H, and temporin-SHf. Anoplin and temporin-SHf demonstrated broad-spectrum microbicidal activity against Gram-positive and Gram-negative bacteria and yeasts, with low hemolytic activity. However, temporin-H contains no antibacterial activity, but it can act synergistically against Gram-negative strains when combined with classical antibiotics. Based on this data, anoplin and temporin-SHf seem to be possible new anti-infective candidates due to their high potency with small and simple structures. The small and simple structures also provide the advantages for chemical modifications and structure-activity relationship studies as well as for investigating their mode of action, which may be useful for the development of a novel class of antibiotics. However, the too short peptides may illustrate some limitations particularly in chemical modifications because they may contain only their pharmacophores in their structures. In spite of the fact that anoplin and temporin-SHf are attractive to be used as candidates or modification templates for further development in therapeutic or industrial uses due to their properties, potencies, toxicities and commercial feasibilities, further studies such as *in vivo* toxicity, efficacy, pre-formulation, formulation, and stability studies are necessary in order to conclude and confirm that these peptides are appropriate to be realistic promising alternative antibiotics.

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