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A review emphasizing on utility of heptad repeat sequence as a tool to design pharmacologically safe peptide-based antibiotics

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ABSTRACT

Extensive usage of antibiotics has created an unprecedented scenario of the rapid emergence of many drug-resistant bacteria, which has become an alarming public health concern around the globe. Search for better alternatives that are as efficacious as antibiotics led to the discovery of antimicrobial peptides (AMPs). These small cationic amphiphilic peptides have emerged as a promising option as antimicrobial agents, owing to their multifaceted implications against varied pathogens. Recent years have witnessed tremendous growth in research on AMPs resulting in them being tested in clinical trials of which six got approved for topical application. The relatively less successful outcome has been attributed to the poor cell selectivity shown by most of the naturally occurring AMPs. This drawback needs to be circumvented by identifying strategies to design safe and effective peptides. In the present review, we have emphasized the importance of heptad repeat sequence (leucine and/or phenylalanine zipper motif) as a tool that has shown great promise in remodeling the toxic AMPs to safe antimicrobial agents.

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1. Introduction

Prokaryotes and eukaryotes have evolved multiple independent strategies to defend against the invading pathogens and to ward off competition from related organisms, which is essential for their survival. One of the common strategies employed by prokaryotes (bacteria) and eukaryotes (fungi) is to produce bioactive compounds called antibiotics [1,2]. These structurally diverse and small compounds are complex molecules comprising of multiple ring structure or uncommon amino acid residues [3]. Many of these natural antibiotics (ansamycins, chloramphenicol, β-lactams, erythtromycin, glycopeptides, etc) are commonly used in clinical applications for treatment of various bacterial infections [4]. Mechanistically, they commonly target cell wall synthesis (β-lactams like penicillin, cephalosporins etc; glycopeptides like vancomycin, daptomycin etc), protein synthesis (macrolides, tetracyclins, amphenicol, oxazolidinone etc), nucleic acid synthesis (fluoroquinolones, rifamycins) and metabolic pathways (sulfanilamide, p-aminosalicylic acid etc) [5]. Although discovery of antibiotics is deemed a 'magic bullet' for mankind, owing to its immense success in controlling bacterial diseases; the recent decades have seen a rising problem of antimicrobial drug resistance due to emergence of multi-drug resistant (MDR) bacteria or "superbugs" [6]. Two possible ways by which pathogens may acquire resistance is either through *de novo* mutations or through horizontal gene transfer events from fellow pathogens. Drug-resistant strains are able to nullify drug's action through modification of its target site, or by production of drug-destructive enzymes. There are numerous reports which suggest drug-resistant strains either possess over-expressed efflux system for inhibiting cellular penetration of drug or they possess an alternative metabolic pathway to overcome the drug's action [7,8].

While one of the major focuses of scientific community has been search of new targets and testing new antibiotics, recent efforts led to consensus of exploring antimicrobial peptides (AMPs). AMPs are an integral part of host's defense barrier against pathogenic invasions and have been reported in a wide range of species including plants, animals and human beings [9]. Their existence is evolutionary conserved, as revealed by the comparative genomic analyses of several organisms [10,11]. In humans, AMPs are present in fluids of ear, eyes, trachea, lungs, gut and skin as these are the first point of contact with environmental pathogens. They are considered to be the components of the innate immunity, and work as the preliminary non-specific line of defense against various infections in complementation with the highly specific cell-mediated component [12].

Gramicidins were the first naturally occurring AMP isolated from soil bacteria, *Bacillus brevis,* in 1939. They were found to be active against several Gram-positive bacteria but only few Gram-negative bacteria. Owing to severe hemolytic activity and low water solubility, gramicidins were clinically approved only for topical application related to ophthalmological infections [13]. Nonetheless, gramicidins were the first peptide-based antibiotics that were commercially manufactured, which also revolutionized the quest for naturally occurring AMPs or host defense peptides (HDPs), as therapeutic avenues to treat various infections [14]. Furthermore, in 1980s, identification and characterization of cecropins and magainins led to more focus on HDPs as promising antibiotic molecules against infections. Multiple other reports also show the efficacy of AMPs/HDPs in wound-healing, cancer cell signaling, angiogenesis and inflammatory response [15,16]. According to APD (Antimicrobial Peptide Database; http://aps.unmc.edu/AP/main.php), over 3200 unique and effective AMP sequences have been identified or predicted so far from various organisms [17].

Although AMPs target bacteria, their efficacy as a drug molecule is limited owing to the various factors which are outlined in figure-1. The most prominent concern lies in the propensity of AMPs to interact non-specifically with mammalian cells [18]. So, complete understanding of the factors involved in regulating their selectivity towards specific cell types is required. Several studies have elucidated information about the structure-activity relationship of AMPs, still our understanding about the balance between efficacy and toxicity is in the nascent stage [19,20]. Various analogs of naturally occurring AMPs have been created by substitution, shuffling, insertion or deletion of specific amino acid residues to reduce toxicity, without perturbing the amphipathic nature [21]. One such strategy involves modifications of residues in a heptad repeat sequence to achieve the delicate balance between antimicrobial and cytotoxic profiles of naturally occurring AMPs [22–24]. Additional studies highlighted the role of heptad repeat sequence in neutralizing lipopolysaccharide mediated endotoxin effect [25–27]. However, such an effect is out of the scope of the present review, so we have not discussed any study related to it. In this review, we have focused on the details of how sequence modifications of naturally occurring AMPs reduces their cytotoxicity and haematotoxicity, without affecting the antimicrobial properties. In particular, we have discussed the relevance of modification in leucine zipper motif (LZM) and phenylalanine zipper motif (PZM) and explained its implications in designing non-toxic analogs of naturally occurring AMPs.

2. Identification and characterization of heptad repeat sequence

Heptad repeats are structural motifs consisting of repeating pattern of seven amino acids present in coiled coils. Past literature suggests that the knowledge about the heptad repeat sequence was ascertained from the fusion protein of HIV, Influenza, or Sendai virus. Several studies have shown association of LZM with viral fusion proteins and other structural or functional proteins [28,29]. Interestingly, Reitter et al. found that mutation induced in the LZM (leucine at position 481, 488 and 495 substituted with alanine) completely abrogates fusion activity of fusion-protein of Newcastle disease virus [30]. Biophysical studies performed on a 36-residue stretch of Influenza hemagglutinin by Carr and co-workers showed that heptad repeat sequence configures a coiled-coil structure. They further proposed that heptad repeat sequence extends throughout the 88-residues of the fusogenic form of influenza, which forms the trimeric coiled-coil structure in native form [31].

The concept of heptad repeats was more pronounced after the characterization of the LZM in GCN4, a DNA-binding protein, involved in transcription of yeast amino acid biosynthesis genes (figure-2). GCN4 leucine zipper was found to possess a stable α-helical structure in solution [32].

3. Development of non-toxic peptides based on LZM

The LZM is a heptad repeat sequence comprising periodic repetition of leucine at every seventh position in a peptide sequence. As shown in figure- 2, for easy characterization and identification, the seventh position in a peptide sequence is termed 'a' and the subsequent position as 'b' 'c' 'd' 'e' 'f' 'g'. In many of the cases, 'd' position(s) to is occupied by leucine or isoleucine residues. Leucine zippers are an integral part of regulatory proteins of prokaryotes and eukaryotes, but they are predominantly a trademark of eukaryotes [33]. Research over several years has critically shown the utility of LZM in studying protein folding, and protein/peptide engineering [34–36].

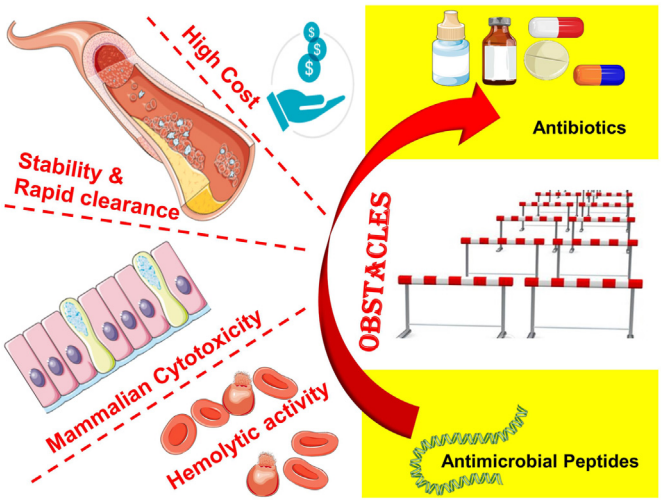

Fig. 1. Factors hindering the use of AMPs as antibiotics.

Study performed by Yadav and co-workers identified for the first time the role of the amphipathic LZM in modulating the cytotoxic effects of proteinaceous toxin (Hemolysin E, HlyE) isolated from a pathogenic strain of *Escherichia coli* [37]. Bacterial strains expressing HlyE were known to lyse erythrocytes and macrophages of several mammalian species including humans. Prior biophysical and biochemical characterization studies of HlyE were unable to determine the mechanism of membrane assembly and the cause of toxicity. To identify the probable membrane interacting domain, Yadav and co-workers identified three heptads (two heptadic positions engaged by leucine & isoleucine and the other two engaged by valine & phenylalanine) in the sequence of hemolysin-E at the C-terminal. They designed and synthesized a wild type peptide (H-205) based on the LZM of HlyE protein and two corresponding peptide mutants (Mu 1-H-205, Mu 2-H-205) by swapping the heptadic leucine/isoleucine with hydrophilic amino acids [37]. Their study revealed that out of the three peptides, wild type peptide attained helical structure in a membrane mimicking environment rather than aqueous environment, highlighting the importance of LZM in designing membrane selective AMPs. Table-1 lists all the sequences of toxic naturally occurring AMPs along with their corresponding non-toxic mutants, which were designed on the concept of LZM.

3.1. Development of non-toxic analogs of melittin

Melittin is a well-studied AMP isolated from the venom of

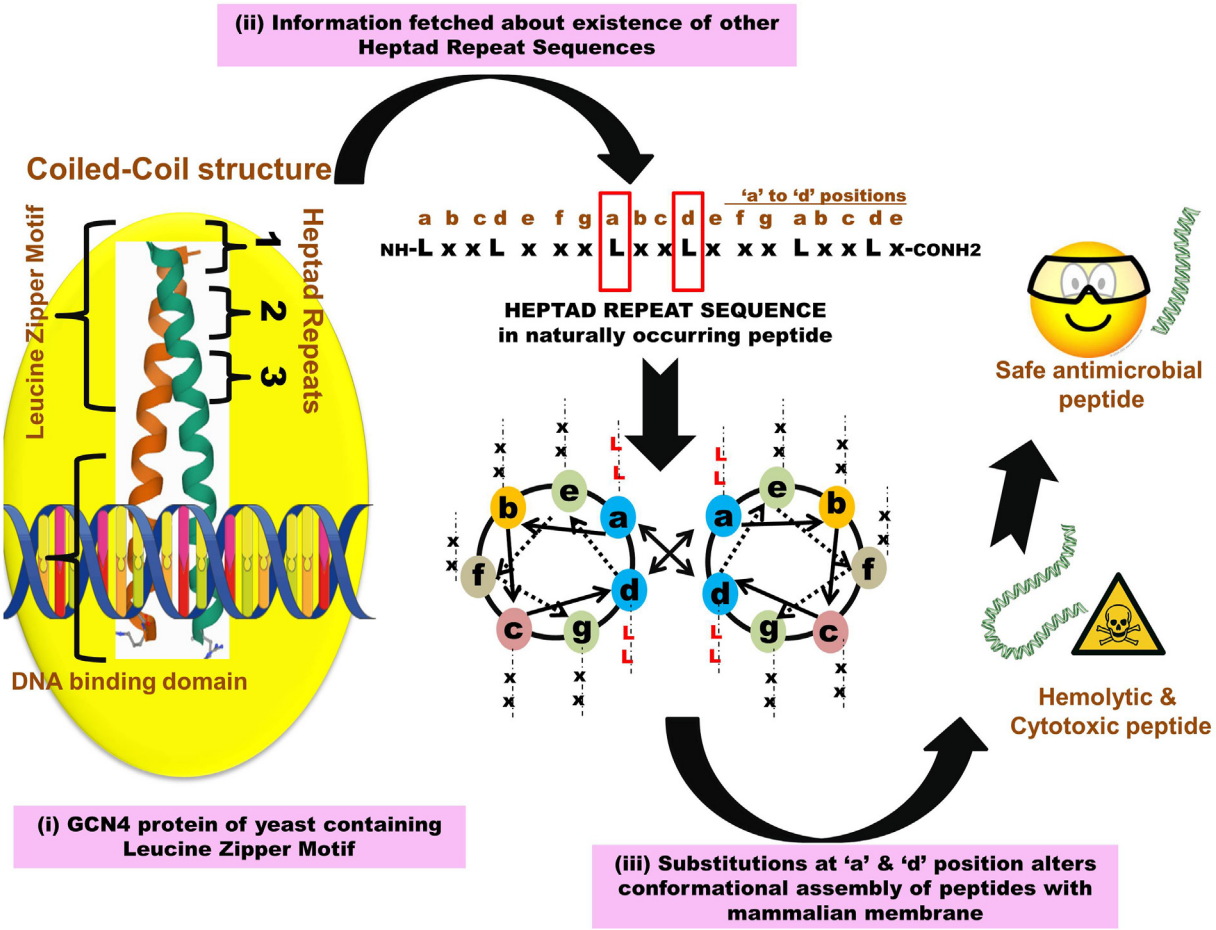

Fig. 2. Schematic overview of the concept of heptad repeat sequence. (i) Identification of leucine zipper motif and its role in protein dimerization was first identified in the yeast protein GCN4. (ii) Characterization and identification of the LZM/PZM in a model peptide sequence. Here every seventh position in a heptad sequence is termed 'a' and the subsequent position as 'b' 'c' 'd' 'e' 'f' 'g'. In many of the cases, 'd' position(s) is occupied by leucine or phenylalanine residue. Helical-wheel projections of the peptide sequence showing interactions between heptadic repeat amino acid positions. Here 'x' means any other amino acid in the peptide sequence. (iii) Substitutions at these heptadic repeats could yield a non-toxic and safe AMP based antibiotic drug.

european bee, *Apis mellifera*. It belongs to the family of cytolytic peptides, commonly involved in the immune response of the organisms [38]. The crystal structure of melittin illustrates its tetrameric structure which is in the form of helix-bend-helix, with two helix encompassing amino residues 1–10 and 13–26 and the bend region encompassing the 11th -and 12th - amino acid residues [39]. Melittin shows analgesic, anti-inflammatory, anti-arthritic and antinociceptive activity at lower doses, but the higher doses tend to be highly hemolytic [40]. Gajski & Garaj-Vrhovac have extensively reviewed the anticancer activity of melittin with mechanistic insights highlighting how melittin is capable of inducing apoptotic death in cancer cells via activation of caspases, matrix metalloproteinases, and perturbations in cell cycle progression. They critically proposed that all the therapeutic effects of melittin get limited due to their toxicity towards normal cells. So, there is a need to modulate its toxicity either by sequence modifications or by

Table 1
List of most well-studied antimicrobial peptides and their respective heptad repeat sequence based mutated analogs. Amino acids at 'a' and 'd' positions (heptadic positions) are in bold font and mutated amino acids are in red colour, bold and underlined font. *Synthetically designed peptides based on heptad repeat sequence. N.D. (Not Determined).

S. No	Peptide Name	Amino acid sequence	% haemolytic activity	Cell viability (% of control)	Ref
1	Melittin	a d a d Melittin: GIGAV**L**KVLTTG**L**PAL**I**SWIKRKRQQ-CONH2 MM-1: GIGAV**L**KVLTTG**A**PAL**I**SWIKRKRQQ-CONH2 MM-2: GIGAV**A**KVLTTG**A**PAL**I**SWIKRKRQQ-CONH2	Melittin: ~ 90% at ≥ 6μM MM-1: ~ 30% at ≥ 6μM MM-2: ~ 2% at ≥ 6μM	N.D.	45
		a d a d a Melittin: GIGAV**L**KV**L**TTG**L**PAL**I**SWIKRKRQQ-CONH2 L9A: GIGAV**L**KV**A**TTG**L**PAL**I**SWIKRKRQQ-CONH2 L16A: GIGAV**L**KV**L**TTG**L**PA**A**ISWIKRKRQQ-CONH2 L916A: GIGAV**L**KV**A**TTG**L**PA**A**ISWIKRKRQQ-CONH2 L913A: GIGAV**L**KV**A**TTG**A**PAL**I**SWIKRKRQQ-CONH2	Melittin: ~ 90% at ≥ 7μM L9A: ~ 10% at ≥ 7μM L16A: ~ 10% at ≥ 7μM L916A: ~ 1% at ≥ 7μM L913A: ~ 1% at ≥ 7μM	N.D.	47
		a d a d a ME: GIGAV**L**KVLTTG**L**PAL**I**SWIKRKRQQ-CONH2 ME-D: GIGAV***L***KVLTTG***L***PALISW***I***KRKRQQ-CONH2 (***L***, ***I*** represents D-amino acids)	ME: 10% at 0.78μM ME-D: 10% at 6.25 μM	N.D.	48
		a d a d a ME: GIGAV**L**KV**L**TTG**L**PAL**I**SWIKRKRQQ-CONH2 ME-a: GIGAV***a***KV**L**TTG***a***PAL**I**SW***a***KRKRQQ-CONH2 ME-l: GIGAV***l***KV**L**TTG***l***PAL**I**SW***l***KRKRQQ-CONH2 ME-f: GIGAV***f***KV**L**TTG***f***PAL**I**SW***f***KRKRQQ-CONH2 ME-k:GIGAV***k***KV**L**TTG***k***PAL**I**SW***k***KRKRQQ-CONH2 (***a***, ***l***, ***f***, ***k*** represents peptoid residues)	ME: 100% at ≥ 10μM ME-a: Nothing even at100 μM ME-l: Nothing even at100 μM ME-f: Nothing even at100 μM ME-k: Nothing even at100 μM	ME: 5% at 100μM ME-a: 90% at 100 μM ME-l: 90% at 100 μM ME-f: 90% at 100 μM ME-k: 90% at 100 μM (Tested in NIH 3T3 & HeLa cells)	50
2	BMAP-28	→ ← a d a d a d a BMAP-28: GG**L**RSLGRKILRAWKK**Y**GP**I**IVPII**R**I-CONH2 Mu-1: GG**A**RSLGRKILRAWKK**Y**GP**I**IVPII**R**I-CONH2 Mu-2: GG**L**RSLGRK**A**LRAWKK**Y**GP**I**IVPII**R**I-CONH2 Mu-3: GG**L**RSLGRKILRAWKK**Y**GP**A**IVPII**R**I-CONH2 Mu-4: GG**A**RSLGRK**A**LRAWKK**Y**GP**I**IVPII**R**I-CONH2 Mu-5: GG**A**RSLGRK**A**LRAWKK**Y**GP**A**IVPII**R**I-CONH2	BMAP-28: ~ 60% at ≤ 20μM Mu-1: ~ 40% at ≤ 20μM Mu-2: ~ 20% at ≤ 20μM Mu-3: ~ 10% at ≤ 20μM Mu-4: ~ 15% at ≤ 20μM Mu-5: ~ 2% at ≤ 20μM	BMAP-28: ~ 10% at ≥ 20μM Mu-1: ~ 20% at ≥ 20μM Mu-2: ~ 20% at ≥ 20μM Mu-3: ~ 75% at ≥ 20μM Mu-4: ~ 65% at ≥ 20μM Mu-5: ~ 95% at ≥ 20μM (Tested in NIH 3T3 cells)	23
		a a a a BMAP-28: GG**L**RSLGRK**I**LRAWKKYGP**I**IVPII**R**I-CONH2 BMAP-28M: GG**L**RSLGRK**I**LRAWKKYG**IP**IVPII**R**I-CONH2	BMAP-28: ~60% at ≤ 20μM BMAP-28M: ~10% at ≤ 20μM	N.D.	55
3	BMAP-27	a d a d a d BMAP-27: GR**F**KR**F**RKK**F**KKLFKKLSPVIPLLHL-CONH2 Mu-1: GR**F**KR**F**RKK**A**KKLFKKLSPVIPLLHL-CONH2 Mu-2: GR**F**KR**F**RKK**F**KKLFKK**A**SPVIPLLHL-CONH2 Mu-3: GR**A**KR**F**RKK**A**KKLFKKLSPVIPLLHL-CONH2 Mu-4: GR**F**KR**F**RKK**F**KK**A**FKK**A**SPVIPLLHL-CONH2	BMAP-27: ~ 25% at ≤ 50μM Mu-1: ~ 2% at ≤ 50μM Mu-2: ~ 2% at ≤ 50μM Mu-3: ~ 2% at ≤ 50μM Mu-4: ~ 2% at ≤ 50μM	BMAP-27: ~10% at ≤ 50μM Mu-1: ~70% at ≤ 50μM Mu-2: ~70% at ≤ 50μM Mu-3: ~65% at ≤ 50μM Mu-4: ~80% at ≤ 50μM (Tested in NIH 3T3 cells)	54
4	IsCT	a d a d IsCT:I**L**GK**I**WEG**I**KS**L**F-CONH2 E7K-IsCT:I**L**GK**I**W**K**G**I**KS**L**F-CONH2 I9K-IsCT:I**L**GK**I**WEG**K**KS**L**F-CONH2 E7K,I9K-IsCT:I**L**GK**I**W**K**G**K**KS**L**F-CONH2	IsCT: ~ 90% at ≤ 100μM E7K-IsCT: ~ 50% at ≤ 100μM I9K-IsCT: ~ 1% at ≤ 100μM E7K,I9K-IsCT:~1% at ≤100μM	IsCT: ~ 10% at 25μM E7K-IsCT: ~ 25% at 25μM I9K-IsCT: ~ 95% at 25μM E7K,I9K-IsCT:~95% at 25μM (Tested in murine 3T3 cells)	61

5	Piscidin-1	a d a d a d Piscidin-1: FFHHIFRGIVHVGKTIHRLVTG-CONH2 I5A- Piscidin-1: FFHHAFRGIVHVGKTIHRLVTG-CONH2 I9A- Piscidin-1: FFHHIFRGAVHVGKTIHRLVTG-CONH2 I16A- Piscidin-1: FFHHIFRGIVHVGKTAHRLVTG-CONH2 I5V-Piscidin-1: FFHHVFRGIVHVGKTIHRLVTG-CONH2 I9V-Piscidin-1: FFHHIFRGVVHVGKTIHRLVTG-CONH2 I16V-Piscidin-1: FFHHIFRGIVHVGKTVHRLVTG-CONH2 I5F,F6A-Piscidin-1: FFHHFARGIVHVGKTIHRLVTG-CONH2 V12I-Piscidin-1: FFHHIFRGIVHIGKTIHRLVTG-CONH2	Piscidin-1: 100% at 50μM I5A- Piscidin-1: 2% at 50μM I9A- Piscidin-1: 20% at 50μM I16A- Piscidin-1: 2% at 50μM I5V-Piscidin-1:70 % at 50μM I9V-Piscidin-1: 70% at 50μM I16V-Piscidin-1:70 % at 50μM I5F,F6A-Piscidin-1: 20% at 50μM V12I-Piscidin-1:100% at 50μM	Piscidin-1: 10% at 50μM I5A- Piscidin-1: 80% at 50μM I9A- Piscidin-1: 60% at 50μM I16A- Piscidin-1: 90% at 50μM I5V-Piscidin-1: 25% at 50μM I9V-Piscidin-1: 20% at 50μM I16V-Piscidin-1: 30% at 50μM I5F,F6A-Piscidin-1: 50% at 50μM V12I-Piscidin-1: 10% at 50μM (Tested in murine 3T3 cells)	24
6	Clavaspirin	a d a d Clavaspirin: FLRFIGSVIHGIGHLVHHIGVAL-CONH2 CSP-1:FLRFAGSVIHGAGHLVHHIGVAL-CONH2 CSP-2:FLRFKGSVIHGKGHLVHHIGVAL-CONH2 CSP-3:FKRFIGSVKHGIGHLVHHIGVAL-CONH2 CSP-4:FLRFIGSVKHGKGHLVHHIGVAL-CONH2	Clavaspirin: 50% at 64μM CSP-1: 50% at 334μM CSP-2: 50% at > 400μM CSP-3: 50% at > 400μM CSP4: 50% at > 400μM	Clavaspirin: 50% at 247μM CSP-1: 50% at > 400μM CSP-2: 50% at > 400μM CSP-3: 50% at 188μM CSP4: 50% at > 400μM (Tested in HaCaT cells)	69
7	Pseudin-2	Zipper-1 a a a zipper-2 a a a Pseudin-2: GLNALKKVFQGIHEAIKLINNHVQ-CONH2 P2-LZ1: GLNAAKKVFQGAHEAIKLINNHVQ-CONH2 P2-LZ2: GANALKKVAQGIHEAIKLINNHVQ-CONH2 P2-LZ3: GLNALKKVFQGAHEAIKLANNHVQ-CONH2 P2-LZ4: GLNALKKVAQGIHEAAKLINNHVQ-CONH2 P2-LZ5: GLNALKKVKQGKHEAIKLINNHVQ-CONH2	None of the mutant analog exhibited haemolytic activity as assessed by digital recording of the tubes	Pseudin-2: 20% at 256μM P2-LZ1: 20% at 256μM P2-LZ2: 100% at 256μM P2-LZ3: 100% at 256μM P2-LZ4: 100% at 256μM P2-LZ5: 100% at 256μM	74
8	Temporin L	a d a TempL: FVQWFSKFLGRIL-CONH2 F5A-TempL: FVQWASKFLGRIL-CONH2 F8A-TempL: FVQWFSKALGRIL-CONH2 F5,8A-TempL: FVQWASKALGRIL-CONH2 F5L-TempL: FVQWLSKFLGRIL-CONH2 F8L-TempL: FVQWFSKLLGRIL-CONH2 F5,8L-TempL: FVQWLSKLLGRIL-CONH2	TempL: 83% at 24μM F5A-TempL: 6.8% at 24μM F8A-TempL: 13% at 24μM F5,8A-TempL: 4.3% at 24μM F5L-TempL: 61% at 24μM F8L-TempL:44.7% at 24μM F5,8L-TempL: 98% at 24μM	TempL: 50% at 24μM F5A-TempL: 27% at 24μM F8A-TempL: 35% at 24μM F5,8A-TempL: 18% at 24μM F5L-TempL: 50% at 24μM F8L-TempL: 45% at 24μM F5,8L-TempL: 50% at 24μM (Tested in murine 3T3 cells)	26
9	LZP *	a d a d a LZP:LKALKKALKWLKKALKALKKA-CONH2 LZP(L4A):LKAAKKALKWLKKALKALKKA-CONH2 LZP(L8A):LKALKKAAKWLKKALKALKKA-CONH2 LZP(L11A):LKALKKALKWAKKALKALKKA-CONH2 LZP(L4A/L8A):LKAAKKAAKWLKKALKALKKA-CONH2 LZP(L4A/L11A):LKAAKKALKWAKKALKALKKA-CONH2 LZP(L8A/L11A):LKALKKAAKWAKKALKALKKA-CONH2	LZP: 30% at 30μM LZP(L4A): 10% at 30μM LZP(L8A): 10% at 30μM LZP(L11A): 13% at 30μM LZP(L4A/L8A): 2% at 30μM LZP(L4A/L11A): 2% at 30μM LZP(L8A/L11A): 2% at 30μM	N.D.	85
10	SLZP, ASA, DLSA, PSA *	a d a d SLZP:LRRLLRWLRRLLRR-CONH2 ASA:LRRAARWARRLLRR-CONH2 DLSA:LRRLLRWLRRLLRR-CONH2 PSA:LRRPPRWPRRLLRR-CONH2 (L represents D-amino acids)	SLZP: ~ 80% at ≥ 70μM ASA: ~ 2% at ≥ 70μM DLSA: ~20% at ≥ 70μM PSA: ~ 1% at ≥ 70μM	SLZP: ~ 2% at ≤ 10μM ASA: ~ 80% at ≤ 10μM DLSA: ~ 65% at ≤ 10μM PSA: ~ 85% at ≤ 10μM (Tested in murine 3T3 cells)	86
11	LRP, VRP, FRP, ARP *	a d a d a LRP:LRRLRKWLRRLLKLL-CONH2 VRP:VRRVRKWVRRVLKLV-CONH2 FRP: FRRFRKWFRRFLKLF-CONH2 ARP:ARRARKWARRALKLA-CONH2	LRP: ~ 80% at ≥ 60μM VRP: ~10% at ≥ 60μM FRP: ~70% at ≥ 60μM ARP: ~ 1% at ≥ 60μM	LRP: ~ 1 0% at ≥ 20μM VRP: ~10% at ≥ 20μM FRP: ~70% at ≥ 20μM ARP: ~ 2% at ≥ 20μM (Tested in murine 3T3 cells)	87
12	FR-15 *	a d a d FR-15: FRRFFKWFRRFFKFF-CONH2 FR4P: FRRPFKWFRRFFKFF-CONH2 FR8P: FRRFFKWPRRFFKFF-CONH2 FR11P: FRRFFKWFRRPFKFF-CONH2 FR4,8P: FRRPFKWPRRFFKFF-CONH2 FR8,11P: FRRFFKWPRRPFKFF-CONH2	FR-15: ~ 100% at ≤ 70μM FR4P: ~ 90% at ≤ 70μM FR8P: ~ 5% at ≤ 70μM FR11P: ~ 2% at ≤ 70μM FR4,8P: ~ 2% at ≤ 70μM FR8,11P: ~ 2% at ≤ 70μM	FR-15: ~ 20% at ≤ 50μM FR4P: ~ 30% at ≤ 50μM FR8P: ~ 80% at ≤ 50μM FR11P: ~ 80% at ≤ 50μM FR4,8P: ~ 90% at ≤50μM FR8,11P: ~ 95% at ≤ 50μM (Tested in murine 3T3 cells)	22
13	Magainin-2	a d a d Magainin-2:GIGKFLHSAKKFGKAFVGEIMNS-CONH2 Mag-mut: GGGKALHSFKKFIKAFVGEIMNS -CONH2	Magainin-2: ~ 2% at 50μM Mag-mut: ~16% at 50μM	Magainin-2: ~75% at 100μM Mag-mut: ~10% at 100μM (Tested in murine 3T3 & CHO-K1cells)	90

proper choice of delivery vehicle such as nanoparticle [41].

Over the years, several attempts have been made to incorporate modifications in the original structure to enhance the antimicrobial potential and reduce hemolytic nature. Among various such attempts, one study involved substitution of the first twenty amino acids with another helix forming sequence, which resulted in no significant change in its antimicrobial or hemolytic activity [42]. Other investigators took a different approach where several fusion or hybrid peptides were designed using the wild type sequence of melittin. Few of the hybrid peptides showed non-hemolytic nature, but their specificity towards several but not all bacterial species was discouraging [43]. A major advancement in controlling the hemolytic action of AMP was achieved in the early 1990s by Blondelle et al. when they created several analogs of melittin by deleting leucine, lysine or valine at several locations in the main sequence. Although these analogs had lesser hemolytic activity, but this was also accompanied with reduced antimicrobial activity [44]. Despite the enormous amount of work done on melittin, the reason behind its poor cell selectivity was not well understood till the 2000s.

A significant breakthrough was achieved in 2004, when Asthana and co-workers identified and characterized the presence of LZM in melittin [45]. As shown in table- 1, authors designed and synthesized two mutant analogs of melittin by specifically substituting the heptad repeat leucine at 'a' position singly (MM-1) and doubly (MM-2), with the less hydrophobic alanine residue. Out of the two designed mutant analogs, MM-1 exhibited 10–20% hemolytic activity and MM-2 showed negligible hemolytic activity as compared to the wild type melittin, which exhibited 100% hemolytic activity at 6 μM. Interestingly, both the mutant analogs exhibited comparable antimicrobial activity with that of wild type melittin (Table-2). Adoption of amphipathic α-helical structure is the preliminary step in many peptide-membrane interactions and circular dichroism (CD) spectrometry is the best tool for rapid determination of such secondary structures [46]. Asthana and co-workers in their study used CD spectrometry and found that all the peptides retained their secondary structures in a bacterial membrane-mimicking lipid vesicles, but lost the α-helical structure in a mammalian membrane-mimicking lipid vesicle [45]. Furthermore, authors also showed that MM-1 and MM-2 displayed lower membrane permeability in a mammalian membrane mimicking lipid vesicle (zwitter-ionic) as compared to equal permeability in a bacterial membrane-mimicking lipid vesicle (negatively charged) with respect to wild type peptide [45]. In a follow-up study, the same group further showed that the substitution of leucine with alanine at any position ('a' and/or 'd') within the heptad repeat sequence shows similar reduction in hemolytic activity without perturbing antimicrobial activity [47].

To optimize the cell selectivity of melittin, Zhu et al. developed the diastereomer analog ME-D, wherein the normal amino acids in LZM was substituted with D-amino acids. As shown in table- 1 and table- 2, ME-D displayed 8-fold reduction in hemolytic activity with similar or 2-fold enhanced antibacterial activity as compared to wild type melittin peptide. Previously, it has been shown that the peptoid residues (imino acids) lacking amide proton possess a helix-breaking propensity when introduced into a peptide sequence [48,49]. Based on these findings, Zhu and coworkers developed cell selective melittin analogs by swapping leucine and isoleucine with peptoid residues in LZM of melittin as shown in table- 1 [50]. None of the peptoid containing analog exhibited hemolysis and cytotoxicity against tested cell lines (HeLa, 3T3) at a concentration as high as 100 μM. Additionally, all the designed analogs possessed enhanced therapeutic index and selectivity towards bacterial cells, along with a shift in mechanism of action from membrane disruption to perturbation of intracellular events, showing better efficacy [50].

3.2. Development of non-toxic analogs of cathelicidins

Cathelicidins are cationic, structurally diverse, small AMPs that are an integral part of the immune system of mammalian system. All the cathelicidin peptides share highly conserved N-terminal domain (cathelin; cathepsin-L inhibitor) and C-terminal domain endowing antimicrobial activity. Unlike synthetically synthesized antibiotics, cathelicidins are gene-encoded precursors that, when required, mature into active peptides. Their abundant presence at the site gets triggered by microbial invasion to exhibit antimicrobial activity [51]. Till now, 30 cathelicidins have been identified, of which BMAP-27 and BMAP-28 have been extensively studied. Both of these AMPs are known to possess α-helical structure and have potent antimicrobial activity at physiological or high salt concentrations. Apart from being antibacterial and antifungal, BMAP-28 has also shown potent anti-tumor activity, at low concentrations, by inducing intrinsic-pathway mediated apoptosis [52]. Concentrations above 10 μM (for both the peptides) are deemed to be toxic for mammalian cells because of their poor cell selectivity between prokaryotic and eukaryotic membrane [53]. Owing to the severe hemolytic and cytotoxic activity, both of these AMPs were presumed to be inappropriate candidates for drug development.

Interestingly, in two independent studies, the same group identified the LZM in BMAP-28 and LZM/PZM in BMAP-27, at the N- and C-terminals, respectively [23,54]. To assess the probable role of LZM structural element in BMAP-28, authors designed and characterized several alanine substituted ('a' positions) mutant peptide analogs, as shown in table- 1. All the designed analogs exhibited similar MIC values to that of wild type peptide when tested against several microbial strains, as shown in table- 2. Out of the five designed mutant analogs, Mu-5 peptide, harboring triple leucine/isoleucine substitutions exhibited negligible hemolytic activity (1–2%) as compared to the wild type BMAP-28 (65%) when treated up to 20 μM. Similar non-cytotoxic activity was obtained for the Mu-5 (95%) as compared to the wild type BMAP-28 (10%) when tested against murine 3T3 cells. Authors further showed that mutant analogs of BMAP-28 selectively perturb the membrane organization and polarization of bacterial cells but not of mammalian cells [23]. Additionally, Azmi et al. in a very recent study showed that the modulation of single amino acid residue (isoleucine^{20} shuffled with proline^{19}; here number in superscript represents the position of amino acid in the peptide sequence) in the LZM of BMAP-28 at the C-terminus is sufficient to mask the toxic nature without affecting the antimicrobial properties [55].

In a different study, authors explored the probable role of LZM and PZM structural elements in BMAP-27 by substituting leucine/phenylalanine ('a' and/or 'd' positions) with alanine (table- 1). Similar to the BMAP-28 mutant analogs, all the BMAP-27 mutant analogs also exhibited MIC values similar and appreciable to that of wild type BMAP-27 peptide molecule (table- 2). Results further revealed that all the mutant analogs with single or double alanine substitution in LZM or PZM exhibited the pronounced reduction in hemolytic activity and cytotoxicity against mammalian cells as compared to wild type BMAP-27. CD spectrometry and FRET based experiments showed that all the mutant peptides of BMAP-27 retain their α-helical structure and overall assembly only in negatively charged bacterial membrane mimicking vesicle, but not in PBS or zwitterionic mammalian membrane mimicking lipid vesicles [54]. Conclusively, both these studies hinted that heptad repeat sequence elements (LZM/PZM) is a decisive tool which could render these cathelicidins as an appropriate candidate for drug development in future.

3.3. Development of non-toxic analogs of IsCT

A short AMP with selective activity against microbes is a good option as a lead therapeutic agent for the drug development process [14]. IsCT are short (13 amino acid residue) α-helical AMPs first isolated from scorpion, Opisthacanthus madagascariensis. IsCt was named after the place Isalo in Madagascar, where the scorpion was discovered. IsCT being a component of scorpion venom are highly hemolytic in nature and considered the shortest cytotoxic peptide. Therefore modulation of its toxicity and hemolytic activity is of primary concern before considering it as an antibiotic option. Presently it is used to enhance the resistance of plants from insect predation [56]. Several attempts have been made to design safe analogs of IsCT, but most of them did not meet the desired success. One such study explained that the introduction of bend in the main structure of this peptide is crucial for its selectivity towards bacterial membrane [57,58]. Another study suggested substitution of amino acid residues at 5th and 9th position results in enhancement of antibacterial efficacy along with hemolytic activity [59]. Another complexity in designing safe analogs of IsCT could be attributed to the possession of less number of cationic residues [60].

A significant breakthrough was made when Tripathi et al. identified the LZM in the sequence of IsCT [61]. As shown in table-1, authors designed three mutant analogs, of which one mutant comprised of single substitution of glutamic acid at 7th position with lysine to enhance the net positive charge (E7K-IsCT). Second mutant constituted single substitution of isoleucine at position 9 with lysine at the hydrophobic face of IsCT (E9K-IsCT). This 9th position is also an 'a' position of the identified LZM. The third mutant was designed with double substitution of both the 7th and 9th position amino acids by lysine in order to examine the impact of substitution of positive charge at polar and non-polar face of the molecule (E7K,I9K-IsCT). Results revealed that the single substituted mutants exhibited drastic reduction in their hemolytic and cytotoxic activities whereas, double substituted mutant showed no toxic effect even at higher concentrations [61]. In terms of antibacterial efficacy, E7K-IsCT and E9K-IsCT exhibited similar activity, and doubly substituted analog exhibited ~2–3.5 fold higher activity as compared to parent IsCT (table- 2). These results suggest that the installation of positive charge on polar or non-polar surface of IsCT agonize its antibacterial efficacy. In congruence to the previous studies related to LZM substitutions, authors found that wild type IsCT exhibit maximum permeabilization of the mammalian membrane mimicking lipid vesicles followed by E7-IsCT. E9-IsCT and E7K,I9K-IsCT does not exhibit such a phenotypic effect. Authors found wild type IsCT and E7K-IsCT to exhibit permeabilization of the bacterial mimicking lipid vesicles. Despite significant antimicrobial activity, E9K-IsCT and E7K,I9K-IsCT were unable to permeabilize the bacterial membrane-mimetic lipid vesicles, suggesting existence of an alternate non-membrane lytic antibacterial mechanism. Furthermore, CD spectrometry and advanced microscopy based assays confirmed the non-membranous based mechanism for bacterial cell killing of E9K-IsCT and E7K,I9K-IsCT peptides. Authors in their study further identified that the non-membranous targeting mechanism of E9K-IsCT and E7K,I9K-IsCT against bacteria is due to inhibition of nucleic acid and protein synthesis. This study critically highlights that substitution of amino acid at the 'a' position of IsCT heptad repeat sequence renders its easy translocation through the bacterial membrane [61].

3.4. Development of non-toxic analogs of Piscidin-1

Piscidins are 22-amino acid long AMPs and are found in various species of teleost fish. First cationic AMPs of piscidin family (piscidin-1, piscidin-3) were isolated from the mast cells of fishes *Morone chrysops* × *M. saxatilis* [62]. Being a host-defense-peptide, piscidins are hemolytic in nature and possess potent antimicrobial activity against varied microorganisms such as bacteria, virus, fungi and parasites. Among all piscidins identified so far, piscidin-1 exhibits most significant antimicrobial activity with highest bioavailability, as estimated through pharmacokinetic and pharmacodynamics studies performed in mice models [63]. Another alluring feature of piscidin-1 is its thermostability and retention of antibacterial activity at a high salt concentration [64]. Nuclear magnetic resonance analysis of piscidin-1 revealed that it attains amphipathic α-helical conformation similar to the other studied linear AMPs. Additionally, it was also found to neutralize LPS-induced pro-inflammatory response in macrophage cells [24,65]. Masso-Silva and Diamond have extensively reviewed the immunomodulatory and anticancer activity of piscidin-1 against varied cancer cell lines such as A549, HeLa, HL-60, MDA-MB-231, U937 etc [64]. Piscidin-1 (no hemolytic activity up to 10 μg/ml) is comparatively less hemolytic and cytotoxic than melittin (60% hemolytic activity up to 10 μg/ml), but before harnessing its true therapeutic potential structural modifications are needed to further mask such unwanted effects [62].

Several attempts have been made to design the safe analogs of piscidin-1 [66,67], with most promising results obtained in the study by Kumar and colleagues, wherein they identified a long heptad repeat sequence at the N-terminal region of piscidin-1 from amino acids 2 to 19 [24]. Apart from non-cytotoxic and non-hemolytic activities, authors explored the role of heptad repeat sequence substitution in imparting anti-endotoxin activity of piscidin-1. As shown in table- 1, authors designed six analogs where isoleucine at two 'a' and one 'd' position were replaced with either alanine or valine, respectively (I5A, I9A, I16A, I5V, I9V, I16V-piscidin-1). These substitutions were incorporated to infer the role of hydrophobicity of amino acids at these specific positions as alanine is less hydrophobic and valine has comparable hydrophobicity to isoleucine. An additional analog (I5F, F6A-piscidin-1) was designed in order to assess the role of phenylalanine (known to be involved in self-assembly of peptide) when it is swapped from non-heptad repeat position to a heptad repeat position. Authors found that the analogs with single isoleucine to alanine substitution exhibited stronger reduction in cytotoxicity and hemolytic activity whereas isoleucine to valine substitution analogs exhibited moderate effects. However, all the analogs showed comparable antimicrobial activity to that of parent peptide (table- 2). CD-spectra studies were undertaken to compare the self-assembly of these peptides which showed that native piscidin-1 peptide and I5F,F6A-piscidin-1 analog attain the α-helical structure in zwitterionic lipid vesicles as compared to moderate and low helical content in other substituted peptides. All the peptide analogs exhibited significant and comparable α-helical structure in negatively charged lipid vesicle. To validate these findings, authors further utilized confocal microscopy to assess the cellular localization of piscidin-1 and its respective analogs on bacterial cells and human-RBCs by using the NBD-labelled versions of the peptides. Results revealed all the NBD-labelled peptides to accumulate equally onto the bacterial membrane [24]. However, all the other NBD-labelled analogs, except alanine substituted analogs (I5A, I9A, and I16A-piscidin-1) accumulated well onto the hRBCs, implicating their weak binding on eukaryotic membrane and thus providing proof-of-concept for modification of LZM as a tool to design safe analogs.

3.5. Development of non-toxic analogs of clavaspirin

Clavaspirin, a 23-amino acid residue AMP was serendipitously discovered while cloning based characterization of clavanins, a

Table 2
Minimal Inhibitory Concentrations of antimicrobial peptides and their respective mutant analogs against various microbial strains. Here AF represents *Aspergillus fumigatus*, BC represents *Bacillus cereus*, BS represents *Bacillus subtilis*, BM represents *Bacillus megaterium*, CA represents *Candida albicans*, CN represents *Candida neoformans*, CP represents *Candida parapsilosis*, EC represents *Escherichia coli (¥ is DH5α strain, # is ATCC10536 strain, $ is ATCC25922 strain, ǂ is KCTC1682 strain),* FO represents *Fusarium oxysporum*, KP represents *Klebsiella pneumoniae*, PA represents *Pseudomonas aeruginosa*, SA represents *Staphylococcus aureus*, SE represents *Staphylococcus epidermidis*, SP represents *Salmonella pullorum*, SS represents *Sporothrix schenckii*, ST represents *S. typhimurium*, PA represents *Pseudomonas aeruginosa*, PV represents *Pseudomonas vulgaris,* TB represents *Trichophyton Beigelii,* TM represents *Trichophyton mentagrophyte*. N.D. means Not Determined.

S. No	Peptide Name	Minimal Inhibitory Concentration of peptides against different bacterial strains						Minimal Inhibitory Concentration of peptides against different fungal strains						Ref
1	Melittin		**BS**	**EC¥**	**SA**			N.D.						45
		Melittin:	2.0μM	3.9μM	3.6μM									
		MM-1:	2.4μM	4.5μM	4.3μM									
		MM-2:	1.8μM	4.2μM	3.6μM									
			BS	**EC¥**	**SA**			N.D.						47
		Melittin:	3.9μM	3.8μM	3.6μM									
		L9A:	2.4μM	4.3μM	3.6μM									
		L16A:	3.9μM	4.2μM	4.2μM									
		L9,16A:	6.0μM	4.2μM	4.0μM									
		L9,13A:	> 20μM	4.3μM	> 20μM									
			BS	**EC ǂ**	**PA**	**SA**	**SE**	**ST**	N.D.					48
		ME:	1.0μM	2.0μM	2.0μM	0.5μM	1.0μM	2.0μM						
		ME-D:	0.5μM	1.0μM	1.0μM	0.5μM	0.5μM	1.0μM						
			BS	**EC ǂ**	**PA**	**SA**	**SE**	**ST**	N.D.					50
		ME:	1.0μM	2.0μM	2.0μM	0.5μM	1.0μM	2.0μM						
		ME-a:	4.0μM	16.0μM	8.0μM	16.0μM	32.0μM	32.0μM						
		ME-l:	1.0μM	4.0μM	4.0μM	4.0μM	4.0μM	16.0μM						
		ME-f:	1.0μM	4.0μM	4.0μM	2.0μM	2.0μM	8.0μM						
		ME-k:	2.0μM	4.0μM	4.0μM	2.0μM	2.0μM	4.0μM						
2	BMAP-28		**BS**	**EC ¥**	**SA**			N.D.						23
		BMAP-28:	1.6μM	2.4μM	3.0μM									
		Mu-1:	1.2μM	2.5μM	3.1μM									
		Mu-2:	1.5μM	2.6μM	3.2μM									
		Mu-3:	1.7μM	2.7μM	3.8μM									
		Mu-4:	1.5μM	2.7μM	3.8μM									
		Mu-5:	1.7μM	2.6μM	3.9μM									
			BS	**BM**	**EC #**	**SA**	**PA**			**CA**	**CP**			55
		BMAP-28:	2.0μM	2.5μM	2.5μM	2.8μM	2.0μM	BMAP-28:		11.0μM	1.5μM			
		BMAP-28M:	2.0μM	2.5μM	2.7μM	3.0μM	2.0μM	BMAP-28M:		22.0μM	3.0μM			
3	BMAP-27		**BS**	**EC ¥**	**SA**			N.D.						54
		BMAP-27:	2.6μM	2.8μM	2.7μM									
		Mu-1:	2.7μM	2.9μM	2.8μM									
		Mu-2:	2.7μM	2.9μM	2.8μM									
		Mu-3:	2.8μM	2.9μM	2.7μM									
		Mu-4:	2.9μM	2.9μM	2.7μM									
4	IsCT		**BS**	**EC#**	**PA**	**SA**		N.D.						61
		IsCT:	3μM	3μM	4μM	2μM								
		E7K-IsCT:	2μM	2μM	3μM	2μM								
		I9K-IsCT:	2μM	4μM	8μM	8μM								
		E7K,I9K-IsCT:	1μM	1μM	2μM	1μM								
5	Piscidin-1		**BS**	**EC$**	**KP**	**PA**	**SA**	N.D.						24
		Piscidin-1:	8μM	3μM	3.8μM	6μM	1.2μM							
		I5A- Piscidin-1:	16μM	10μM	12μM	20μM	5μM							
		I9A- Piscidin-1:	8μM	5μM	6μM	10μM	2.5μM							
		I16A- Piscidin-1:	8μM	6μM	8μM	12μM	2.5μM							
		I5V-Piscidin-1:	8μM	5μM	6μM	12μM	2.5μM							
		I9V-Piscidin-1:	8μM	5μM	6μM	12μM	2.0μM							
		I16V-Piscidin-1:	8μM	5μM	6μM	12μM	2.0μM							
		I5F,F6A-Piscidin-1:	16μM	10μM	12μM	24μM	5.0μM							
		V12I-Piscidin-1:	6μM	2μM	3μM	4μM	1.0μM							
6	Clavaspirin		**BS**	**EC $**	**PA**	**SE**	**SA**	N.D.						69
		Clavaspirin:	64μM	64μM	>64μM	32μM	64μM							
		CSP-1:	64μM	8μM	32μM	16μM	64μM							
		CSP-2:	4μM	4μM	16μM	32μM	16μM							
		CSP-3:	16μM	4μM	32μM	8μM	8μM							
		CSP-4:	4μM	4μM	32μM	8μM	8μM							
		(MIC values shown here are at 7.4 pH)												
7	Pseudin-2	N.D.							**AF**	**CA**	**CP**	**FO**	**TB**	74
								Pseudin-2:	64μM	12μM	>64μM	4μM	2μM	
								P2-LZ1:	64μM	8μM	>64μM	4μM	2μM	
								P2-LZ2:	24μM	4μM	48μM	2μM	2μM	
								P2-LZ3:	64μM	8μM	>64μM	4μM	6μM	
								P2-LZ4:	32μM	4μM	48μM	2μM	1.5μM	

(*continued on next page*)

Table 2 (*continued*)

S. No	Peptide Name	Minimal Inhibitory Concentration of peptides against different bacterial strains							Minimal Inhibitory Concentration of peptides against different fungal strains						Ref
									P2-LZ5:	16μM	32μM	>64μM	6μM	8μM	
8	Temporin L		**BS**	**ECS**	**KP**	**PA**	**SA**		N.D.						26
		TempL:	15μM	12μM	15μM	15μM	15μM								
		F5ATempL:	30.4μM	28μM	50μM	>60μM	30μM								
		F8ATempL:	15μM	12μM	30μM	15μM	15μM								
		F5,8ATempL:	>60μM	60μM	>60μM	60μM	60μM								
		F5LTempL:	15μM	6μM	15μM	15μM	15μM								
		F8LTempL:	15μM	12μM	15μM	15μM	15μM								
		F5,8LTempL:	60μM	12μM	30μM	15μM	>60μM								
9	LZP		**BS**	**EC ¥**	**SA**				N.D.						85
		LZP:	5.8μM	7.2μM	5.8μM										
		LZP(L4A):	6.2μM	7.4μM	5.6μM										
		LZP(L8A):	6.7μM	7.4μM	6.2μM										
		LZP(L11A):	6.2μM	6.8μM	6.2μM										
		LZP(L4A/L8A):	6.0μM	6.8μM	6.2μM										
		LZP(L4A/L11A):	6.2μM	7.4μM	6.2μM										
		LZP(L8A/L11A):	7.8μM	7.4μM	6.6μM										
10	SLZP, ASA, DLSA, PSA		**BC**	**EC**	**KP**	**PA**	**SA**			**AF**	**CP**	**SS**	**TM**		86
		SLZP:	3.15μg/ml	25μg/ml	3.15μg/ml	25μg/ml	1.56μg/ml		SLZP:	6.25μg/ml	6.25 μg/ml	3.15 μg/ml	6.25 μg/ml		
		ASA:	3.15μg/ml	25μg/ml	6.25 μg/ml	25μg/ml	3.15 μg/ml		ASA:	50μg/ml	12.5μg/ml	12.5μg/ml	100μg/ml		
		LSA:	3.15 μg/ml	12.5μg/ml	6.25 μg/ml	12.5μg/ml	3.15μg/ml		LSA:	6.25μg/ml	12.5μg/ml	6.25μg/ml	12.5μg/ml		
		PSA:	>100μg/ml	25 μg/ml	12.5 μg/ml	100μg/ml	12.5 μg/ml		PSA:	>100μg/ml	>100μg/ml	100μg/ml	>100μg/ml		
11	LRP, VRP, FRP, ARP		**BS**	**EC #**	**PA**	**SA**	**SE**	**KP**		**AF**	**CA**	**CN**	**CP**	**SS**	87
		LRP:	10.5μM	6.2μM	11.2μM	6.2μM	12.1μM	6.2μM	LRP:	3.1μM	3.1μM	6.1μM	3.0μM	1.5μM	
		VRP:	6.8μM	3.0μM	3.2μM	12.5μM	6.0μM	3.2μM	VRP:	8.4μM	9.6μM	8.4μM	1.5μM	6.0μM	
		FRP:	11.3μM	6.0μM	25μM	25μM	13.2μM	6.0μM	FRP:	3.0μM	3.1μM	6.1μM	3.0μM	3.0μM	
		ARP:	20.0μM	4.5μM	6.2μM	>25μM	8.0μM	25μM	ARP:	>25μM	>25μM	>25μM	25μM	25μM	
12	FR-15		**BS**	**ECS**	**KP**	**PA**	**SA**			**AF**	**CA**	**CN**	**SS**	**TM**	22
		FR-15:	3.8μM	6.5μM	7.0μM	15.3μM	14μM		FR-15:	2.1μM	2.1μM	2.1μM	2.1μM	4.4μM	
		FR4P:	1.9μM	4.5μM	3.6μM	15μM	5μM		FR4P:	18μM	2.1μM	2.1μM	9μM	18μM	
		FR8P:	1.9μM	4.5μM	3.0μM	7.7μM	5μM		FR8P:	>35μM	9μM	9μM	35μM	>35μM	
		FR11P:	1.9μM	4.5μM	4.0μM	6.4μM	5μM		FR11P:	35μM	9μM	9μM	9μM	35μM	
		FR4,8P:	2.1μM	4.8μM	5.1μM	32μM	2μM		FR4,8P:	>35μM	9μM	18μM	18μM	>35μM	
		FR8,11P:	2.2μM	4.8μM	6.7μM	10μM	7.1μM		FR8,11P:	>35μM	18μM	18μM	9μM	>35μM	
13	Magainin-2		**BS**	**BM**	**EC #**	**SA**			N.D.						90
		Magainin-2:	10.0μM	12.0μM	5.0μM	40.0μM									
		Mag-mut:	10.0μM	12.0μM	5.0μM	40.0μM									

family of five histidine-rich AMP from marine tunicate *Styela clava* [68]. Clavaspirin demonstrates significant antimicrobial properties against Gram-positive and Gram-negative bacteria at acidic pH, but loses its antimicrobial properties at neutral pH. Furthermore, clavaspirin (IC_{50} = 10 μg/ml) exhibit hemolytic activity against human and bovine RBCs similar to that of melittin (IC_{50} = approximately 5 μg/ml) [68]. In 2018, Lee et al. identified the presence of LZM in the sequence of clavaspirin [69]. As shown in table- 1, authors designed its four analogs, of which the two mutants comprise of double isoleucine$^{5, 12}$ substitutions (both at 'd' positions) with either alanine (CSP-1) or with lysine (CSP-2). The third mutant, CSP-3, was designed by substituting leucine^{2} and isoleucine^{9} (both are 'a' position) with lysine. The fourth mutant, CSP-4, was designed by doubly substituting isoleucine$^{9, 12}$ with lysine (one 'a' and another 'd' position; here number in the superscript represents the position of amino acid in the peptide sequence). The results proclaimed that out of these four analogs, CSP-4 peptide exhibit lowest hydrophobicity, cytotoxicity and hemolytic activity. Excitingly, CSP-4 exhibits significant antimicrobial properties against normal and drug-resistant bacterial strains even at neutral pH (table- 2). Additional experiments demonstrated the inability of microbes to develop resistance against these designed analogs as compared to the conventional antibiotics. MIC values of all the designed analogs against *S. aureus* increased only two times as compared to antibiotics such as daptomycin (>1000 times) or linezolid (>200 times) after 29 passages. CD-spectra studies showed that all the peptides attained random coiled structure in aqueous environment at pH 5.5/7.4, whereas CSP-2, -3, -4 attain alpha-helical structure in a membrane-mimetic environment at pH 7.4 suggesting their efficacious nature compared to natural clavaspirin. Electron and confocal microscopic examinations showed that the antibacterial mechanism of the designed peptides lies in their propensity to damage the membrane [69]. Authors in their study finally concluded that CSP-4 could be developed as an antibiotic drug to treat dermatitis, as assessed through the hairless mouse model of dermal infection [69].

3.6. Development of non-toxic analogs of Pseudin-2

Olson and colleagues isolated a family of four structurally related AMPs (pseudin 1-4) from the skin extract of paradoxical frog, *Pseudis paradoxa*. Out of the four peptides, pseudin-2 was more abundant and exhibited potent antimicrobial activity against Gram-negative bacteria [70]. Another report further suggested that pseudin-2 forms α-helical structure while interacting with bacterial or fungal membrane and subsequently forms pore. Inside the cell, pseudin-2 electrostatically binds to RNA and inhibits protein synthesis [71]. Abdel-wahab and co-authors showed pseudin-2 to stimulate insulin secretion in a calcium-independent manner based on which it was suggested to be looked upon as an insulinotropic drug for type-2 diabetes treatment in future [72]. In an attempt to design peptide-based antimicrobial biomaterials, Kang and

coauthors developed a synthetic and truncated version of pseudin-2 peptide called Pse-T2 [73].

In line with the previous successful attempts of developing non-toxic analogs of naturally occurring AMPs based on LZM, Park and colleagues recently developed several non-toxic analogs of pseudin-2 [74]. Authors identified two heptad repeat sequences within the same frame of pseudin-2 that were occupied by leucine, isoleucine and phenylalanine residues. As shown in table- 1, authors designed four mutated analogs (P2-LZ1, P2-LZ2, P2-LZ3, P2-LZ4) by replacing leucine, isoleucine and/or phenylalanine with alanine designated at 'a' position. Fifth mutant analog, P2-LZ5, was designed by substituting phenylalanine ('a' position) and isoleucine ('d' position) with lysine in order to inhibit and assess self-assembly of the peptide [74]. As shown in table- 2, authors found all the mutant analogs to exhibit significantly lower MICs against yeast as compared to mold fungi at acidic pH. None of the mutant peptide exhibit detectable hemolytic activity when treated up to 64 μM. Except P2-LZ1, none of the mutant peptide showed cytotoxicity against HaCaT (human immortalized keratinocytes) cells when treated up to 256 μM for 24 h. CD spectra revealed that except P2-LZ5, all the mutant analogs attained more helical structure than parent pseudin-2 in the presence of vesicles as compared to the random coiled structure in aqueous solution [74]. Confocal microscopy of rhodamine-labelled peptides showed that all the peptides except P2-LZ5, accumulated on the fungal membrane suggesting a membrane-active mechanism. P2-LZ5 mutant analog was also found accumulated in the cytoplasm, suggesting its different mode of action. Furthermore, non-membranous targeting mechanism of P2-LZ5 against fungi was attributed to its ability to bind to the mitochondrial membrane which resulted in mitochondrial ROS generation and perturbation in membrane potential. Authors in their study finally concluded that P2-LZ4 could be developed as an anti-fungal drug, as assessed through the *C. tropicalis* skin infection mouse model [74].

4. Development of non-toxic peptides based on PZM

The phenylalanine zipper motif (PZM) is a heptad repeat sequence comprising periodic repetition of phenylalanine at every seventh position in a peptide/protein sequence. PZM was first identified to be involved in the dimerization of APS, SH2−B, and Lnk, all of which belongs to the conserved adaptor protein family expressed in mast cells [75]. Later studies showed that leucine/phenylalanine zipper sequence containing peptides interact with bacterial membrane in a stable manner by forming a hydrophobic interphase between the amphipathic helices of coiled-coil [76,77]. Srivastava et al. in their study explored the critical role of PZM in imparting toxic effect of temporin L [26]. Temporin family members are small amphipathic α-helical peptides, effective against varied pathogens such as bacteria, fungi, protozoa, yeast, etc. Few temporin family peptides exhibit chemotactic activities and possess immunomodulatory effects [78]. Temporin L was among the ten temporin family of peptides isolated from the European red frog, *Rana temporaria* and exhibited the highest antimicrobial potency against Gram-positive and Gram-negative bacteria (lethal concentration ranges 0.3−17 μM) as compared to other members which are effective only against Gram-positive bacteria (lethal concentration ranges 2.8−360.0 μM) [79]. Additionally, they have the strongest affinity for lipid membranes and have potential anti-endotoxin property. Rinaldi et al. in their study showed that temporin L is equally effective against cancer cell lines and they perturb the integrity of neutral and negatively charged membranes [80].

Previously it was reported that temporin L is highly hemolytic in nature (80% hemolysis at 8 μM) as compared to its other family members (80% hemolysis more than 20 μM) [80,81]. Srivastava and coworkers identified PZM in the sequence of temporin L and designed six analogs, where phenylalanine at single 'a' or 'd' position was replaced with either alanine (F5A-TempL, F8A-TempL) or leucine (F5L-TempL, F8L-TempL). Additional analogs (F5,8A-TempL; F5,8L-TempL) were designed with double phenylalanine substitutions [26]. In congruence to the LZM substitutions, authors found phenylalanine to alanine substitutions in PZM drastically reduced cytotoxicity and hemolytic activity (table- 1). However, augmentation in hemolytic and anti-endotoxin activity was observed in phenylalanine to leucine substituted analogs. Authors further found all the analogs to possess comparable antimicrobial properties to that of temporin L, except F5,8A-TempL, which exhibit slight reduction in antimicrobial activity (table- 2) [26]. Reduction in antimicrobial activity of F5,8A-TempL is similar to that observed by Pandey et al. in double leucine to alanine substitutions in LZM of melittin [47]. Such a reduction in antimicrobial properties could be attributed to the reduction in hydrophobicity of peptides due to double substitutions. So these results strongly suggests that phenylalanine heptad repeat sequence like leucine heptad repeat can be exploited as a tool in designing safe antimicrobial peptide molecule.

5. Development of synthetic peptides based on heptad repeat sequence

Apart from the technical difficulty of toxicity, the high cost of peptide synthesis is another hurdle, as large amount of peptides are required during pre-and post-clinical trials. So, there is a need for transition towards smaller and less expensive peptides compared to synthesizing and testing larger naturally occurring peptides [82]. To date, most peptides of therapeutic importance have evolved from the structure-function based studies [21]. Structure-assisted designing of small synthetic peptides comes from the study done on Leu-Lys-rich peptides [83]. Optimization of such a small peptide laid the foundation stone for designing of other small synthetic peptides. In this regard, Ahmad et al. tried to develop an AMP system by designing and characterizing single or double alanine substituted LZM containing synthetic family of peptides called LZP which consists of 21 amino acids. As shown in table- 2, all the designed synthetic peptides were equally potent in damaging or depolarizing bacterial membrane. Encouragingly, peptides harboring progressive substitutions of leucine to alanine exhibited impaired binding and localization with hRBCs [84,85]. They further designed many shorter length analogs (14 amino acid residues) such as proline synthetic analog (PSA), D-leucine synthetic analog (DLSA), and alanine synthetic analog (ASA), by substituting leucine in all cases. As shown in table- 2, except PSA, all the analogs exhibited no hemolytic or cytotoxic activity with similar antimicrobial activity [86].

In a different study from the same group, authors explored the influence of different hydrophobic amino acids on antimicrobial, cytotoxic and anti-endotoxin properties of synthetic model peptides. They designed, synthesized and characterized four peptides namely, LRP (leucine residue peptide), FRP (phenylalanine residue peptide), VRP (valine residue peptide), and ARP (alanine residue peptide). As shown in table- 2, all the analogs exhibited similar antimicrobial activities. Although valine is equally hydrophobic in nature as compared to leucine or phenylalanine, but still VRP showed much less cytotoxic and hemolytic activity [87]. Interestingly, Ghosh et al. proposed VRP as a potent vaginal contraceptive molecule since their study revealed VRP to possess spermicidal activity without affecting the vaginal microflora [88]. This study suggests a new therapeutic implication of heptad repeat sequence-based peptide designing which needs further investigation in future. A similar approach was used to design FR-15, a 15-residue

cationic synthetic model peptide based on phenylalanine heptad repeat sequence [22]. As shown in table- 1, five analogs of FR-15 were synthesized and characterized by substituting phenylalanine at 'a' and 'd' position with proline, owing to its helix-breaking property and unique imino structure. Apart from antifungal and anti-inflammatory response, all the analogs of FR-15 exhibited stable antibacterial activities even at high salt concentrations, and high temperatures. All the analogs also showed resistance against the activity of human proteases, which is one of the major hurdles in developing AMP based drugs. Additionally, the two non-toxic analogs (FR8P and FR11P) exhibited significant anti-cancer activity against breast cancer cell lines with IC_{50} value of 50 μM. Mechanistically, both the peptides were able to induce apoptosis via mitochondrial membrane targeting of intrinsic pathway [22]. To the best of our knowledge, this is the first report that suggested that substitutions in PZM with proline could yield broad-spectrum AMPs possessing anti-cancer activity while maintaining their stability under harsh conditions.

Recently, Jia et al. in their study obtained a synthetic peptide by connecting two symmetric heptad repeat sequences through a short loop (GG or pG). Out of the seven designed peptides, LR_{pG} peptide exhibited significant stability at high salt, temperature and pH. Authors further analyzed the peptide to confirm high therapeutic index as compared to other conventional AMPs [89]. All these studies hinted that small synthetic peptides could be designed based on heptad repeat sequence that could overcome high production cost problem. In conclusion, all these success stories of heptad repeat sequence-based designing of peptides are highlighting it as an interesting tool in designing multifaceted drugs for the future.

6. Critically opposing case of magainin-2: development of toxic analogs based on LZM

In the sections above, we have discussed about the role of LZM in imparting hemolytic and cytotoxic activity of naturally occurring AMPs and how loss-of-function of this structural element was obtained by substitutions of leucine at 'a' and/or 'd' positions with less hydrophobic amino acids. In a different approach, Pandey et al. demonstrated proof-of-concept support to this idea by optimizing and constructing an analog of magainin-2 in a way to include a LZM in its sequence. In this gain-of-function approach, shuffling was performed only in the hydrophobic amino acids while maintaining the overall amphipathicity of the mutant peptide [90]. Magainins were first isolated by Michael Zasloff from the skin of *Xenopus laevis,* the African clawed frog [91]. The attractive feature of magainin-2 is its low toxicity towards mammalian cells that led to the development of highly potent derivative called pexiganan (MSI-78). Recently, it has successfully completed phase-III study for the treatment of diabetic foot ulcer [92]. Additionally, several studies

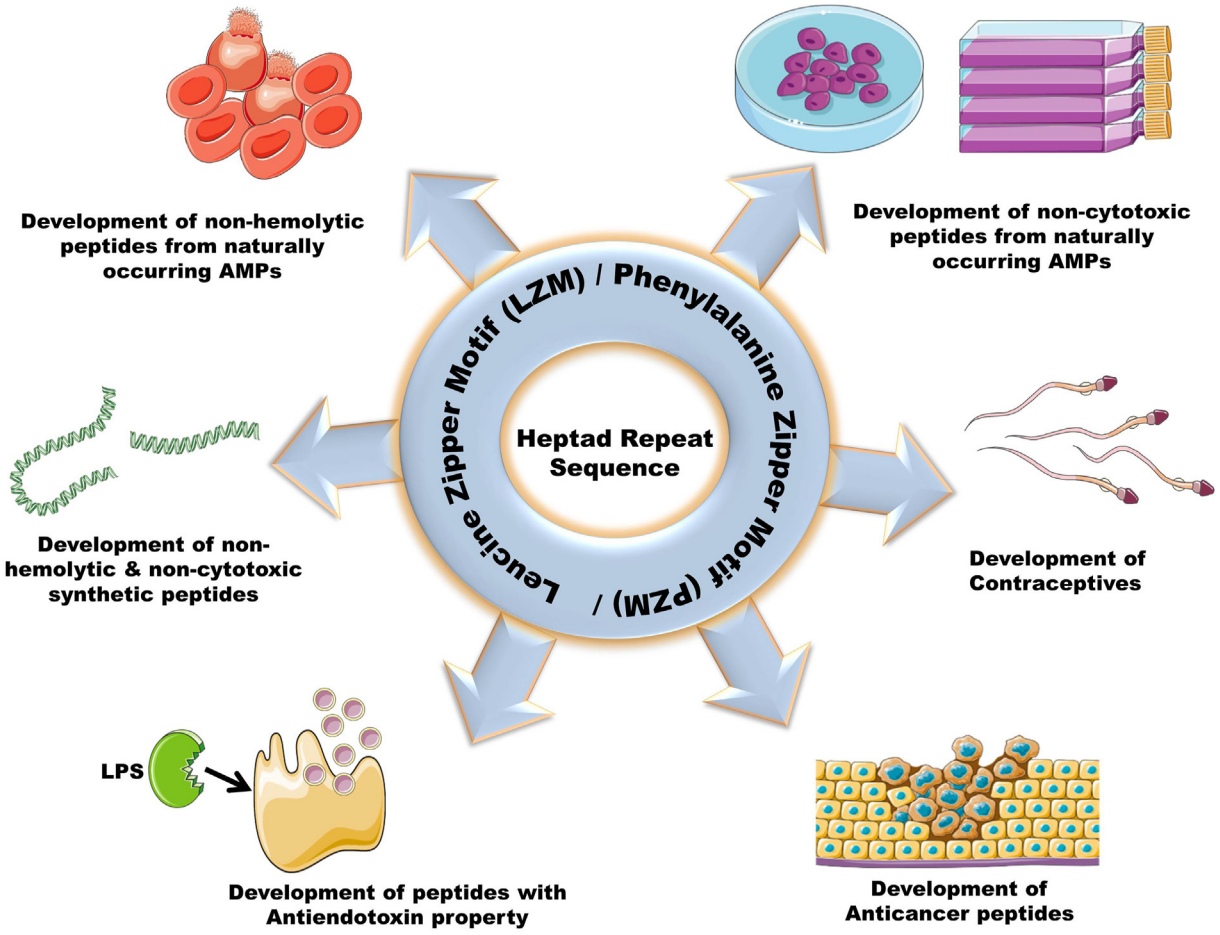

Fig. 3. Schematic overview of the application of heptad repeats (LZM/PZM) in peptide designing. Major implication of LZM/PZM has been postulated in designing non-hemolytic and non-cytotoxic analogs of naturally occurring AMPs and/or designing synthetic AMPs. LZM has been implicated in designing of contraceptive peptides such as in case of VRP and peptides with enhanced anti-endotoxin properties (not discussed in this review). PZM has been implicated in designing peptides with anti-cancer activity such as in case of FR8P and FR11P.

have shown the anticancer activities of magainin-2 against several cancer cell lines [93,94].

Magainins are broad-spectrum AMPs but lack a LZM in its wild type sequence, which possibly contributes to its low toxicity [95]. As shown in table- 1, Pandey and coworkers designed LZM containing magainin mutant by swapping the glycine^{13} and isoleucine^{2} with each other (here number in the superscript represents the position of amino acid in the peptide sequence). An additional swapping of phenylalanine^{5} and alanine^{9} was performed to maintain the hydrophobicity of the mutant peptide [90]. Interestingly, authors found the mutant analog of magainin-2 to exhibit higher hemolytic and cytotoxic against murine 3T3 cells and macrophage RAW264.7, compared to the wild-type peptide. However, both the peptides showed similar antibacterial activity against *E. coli, Bacillus subtilis, Bacillus megaterium, S. aureus* (table- 2). Fluorescent tagging (Rho-labelling) based experiments showed that magainin-mutant possesses a stronger binding affinity towards human erythrocytes as compared to wild type magainin-2. Whereas, both the peptides exhibit similar binding affinity towards the bacterial membrane. Furthermore, CD spectral experiments showed that only the magainin-mutant analog was able to adopt a helical structure in a zwitterionic lipid vesicle. Nonetheless, both these peptides were able to attain the α-helical structure in a negatively charged lipid vesicle [90].

7. Conclusion & future directions

Usage of AMPs has gained immense enthusiasm from academia and more recently from industries owing to their broad-spectrum activity against bacteria, especially multi-drug resistant microbial strains. Interestingly, the U.S. Food and Drug Administration has already approved 60 AMPs and over 150 peptides are currently undergoing clinical trials for testing efficacy in various infections [14]. As of 2020, the peptide drug sale had reached approximately 25billion USD and is estimated to exceed 50billion USD by 2024 (Globenewswire.com; source: Zion market research). Despite the growing business and promising attributes, AMP-based drug discovery is riddled with considerable challenges such as stability, toxicity and cost issues (Figure-1). Moreover, clinical usage of most of the approved AMPs is restricted to topical application only, since the systemic administration of AMPs is still considered to be too toxic for humans. In this review, we have focused on emphasizing the critical role of heptad repeat sequence in governing the toxicity of several naturally occurring AMPs. Compiling all the research studies discussed, we can attribute LZM and PZM as a signature of toxicity. Their presence always aids the toxicity towards human cells and RBCs. Even their inclusion into the non-toxic AMP renders them toxic such as in the case of magain-2. This structural motif provides a critical design strategy for the development of novel and safe AMP moieties showing great potential for clinical use in the near future (Figure-3).

Authors contributions

V.Y. conceived the idea, collected the information, drafted the manuscript, prepared the table and figures. R.M. critically edited and revised the manuscript. Both authors have approved the final version of the manuscript.

Declaration of competing interest

Authors declare no conflict of Interest.

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