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Original Article

The New Insight for Novel Antimicrobial Peptides Designing by Computational Design and Improvement of an Antimicrobial Peptide Derivate of LL-37

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Abstract

Background: LL-37 is one of the well-known antimicrobial peptides which is effective on the extended expecetrome of microbial pathogens. The aim of this study was to design a modified digital analog of LL-37 with enhanced antimicrobial activity and restricted toxicity of the host cell.

Methods: Online databases and software were used for determining LL-37 characteristics such as hydrophobicity, Booman index, and hemolysis probability. Variant structures based on the replacement of leucine and lysine with tryptophan and arginine were calculated as well. Finally, the best sequence was selected and analyzed for approving as an antimicrobial peptide.

Results: The antibacterial characteristics of LL-37 were improved by replacing the arginine and tryptophan and according to systemic calculations, it was defined that this peptide is an antimicrobial peptide by 97% confidential.

Conclusions: In general, bioinformatics tools are considered as one of the most available and efficient tools for antimicrobial peptide designing. Therefore, future studies could use kLL-39 as an antimicrobial peptide and investigate its antimicrobial effects in vitro.

Keywords: LL-37, Antimicrobial peptides, Computational studies, Infectious disease

Background

Small peptides named anti-microbial peptides (AMPs) have antimicrobial effects. Similarly, these peptides almost receive natural sources and are made of and released by different eukaryotic and prokaryotic cells. According to some studies, several artificial AMPs are produced due to their advantages.[1,2] In addition, AMPs are effective on various pathogenic Gram-positive and -negative bacteria and as a part of the innate immune system, these peptides are involved in inflammatory cytokines induction, as well as the activation and motivation of dendritic cells. AMPs occasionally exhibit a chemokine effect and result in the clearance of infection next to the starting of the inflammatory response in the host body.[1,3]

Although their name is antimicrobial peptides, these components are effective not only on bacterial pathogens but also they have anti-biofilm, anti-viral, anti-fungal, anti-cancer, anti-oxidant, anti-inflammatory, and wound therapeutic effects. Today, more than 3000 AMPs are recognized out of which 17 cases (e.g., pexiganan, omiganan, and LL-37) are used in clinical trials.[2,4]

According to previous microbial research, there exists a crisis regarding antibiotic resistance. Further, the emergence and extension of infections with Penicillin-resistant *Streptococcus pneumonia* (PRSP), methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant Staphylococcus aureus (VRSA), penicillin resistant *Neisseria gonorrhoeae* (PRNG), multi-drug resistance (MDR) gram-negative bacteria, totally drug-resistant (TDR) gram-negative bacteria, MDR- tuberculosis (TB), and extensively drug resistance (XDR)-TB strains result in serious concerns about health care systems for the treatment of drug-resistant infections.[5]

Antibiotic resistance crisis is regarded as a serious issue insofar as the World Health Organization named the year 2011 as antimicrobial resistance. According to the growing expansion of antimicrobial resistance, scientists are looking for new antimicrobial components in order to extinct and cure drug-resistant infections. Nowadays, numerous components such as nano-particles, herbal extracts, bacteriophages, and especially AMPs are the candidates for the improvement of current antibiotics.[6-8] AMPs mostly

have positive net charge while bacterial cell membrane (BCM) has a negative charge (Figure 1). Furthermore, AMPs bind to BCM and produce pores and finally block the phospholipids on BCM. Then, net formation by AMPs results in cytoplasmic leakage and cell death.[9] Moreover, AMPs can inhibit RNA and protein synthesis and RNA accumulation inside the bacterial cell and thus kill the cell.[10]

LL-37 (CAP-18) is an 18 kDa peptide from cathelicidins family and contains 37 amino acid repetitions in an α-helical structure. Additionally, LL-37 is produced in macrophages, polymorphonuclear leukocytes, and epithelial cells and vitamin D is an important factor for the expression of LL-37.[11] Several previous studies approved that dialysis patients with a higher level of LL-37 are more resistant to infections than the other patients with a lower level of this peptide, and therefore, the mortality rate is lower in patients of the second group. However, evidence suggests that LL-37 can accelerate Alzheimer's disease progression.[12-14]

The large-scale production of AMPs causes some problems such as low bioavailability, cell toxicity, allergenic reactions, and expensive production price.[15] Therefore, the bioinformatics improvement of AMPs is one of the most important tools for an early analysis of these peptides.[2] Using the bioinformatics analysis of synthetic peptides, researchers can anticipate and optimize the peptide properties and launch their laboratory studies.[2,16] According to some studies, the presence of arginine in the peptide facilitates the penetration of the peptide into the cell. Additionally, the presence of tryptophan increases stability and induces the formation of circular structures in the peptide, which enhances the antimicrobial effects of AMPs.[17,18]

Based on available sources, the LL-37 may have an effect on the onset or progression of Alzheimer's disease. In order to further increase the antimicrobial activity of LL-37 peptide, this study sought to design a new analogue of LL-37 peptide (mLL-37). In other words, mLL-37 was designed to remove the similar structures of β-amyloids that are involved in the pathogenesis of Alzheimer's disease. In addition, some changes in peptide amino acid LL-37 increase its antimicrobial properties as well.

Materials and Methods

In the present study, the amino acid sequencing of antimicrobial peptide LL-37 from the Antimicrobial Peptide database (http://aps.unmc.edu/AP/main.php) was first obtained by Aligned via online ClustalW2 software (http://aps.unmc.edu/AP/main.php).

Analog mLL-37 Peptide Synthesis

It is possible to eliminate β-amyloid structures and prevent Alzheimer disease by applying some modifications such as amino acid racemization for polyglutamine, amino acid removal, and changes in post-translation modifications including glycosylation. In addition, the replacement of the amino acids of arginine (R) and tryptophan (W) instead of lysine (K) and leucine (L) not only changes the net charge of the peptide but also enhance its antibacterial properties.[19,20] According to the above-mentioned explanations, some attempts were made to remove the structures involved in Alzheimer's pathogenesis from the LL-37 peptide and replace the arginine and tryptophan amino acids with lysine and leucine.

The overall profile of the peptide was identified by using online server APD3-AMP tools (http://aps.unmc.edu/AP/tools.php). Further, the potential of binding to the protein or "Boman index" evaluated by utilizing the above-mentioned server (http://aps.unmc.edu/AP/prediction/prediction_main.php) (Figure 2). It should be noted that the Boman index is based on the sum of solubility

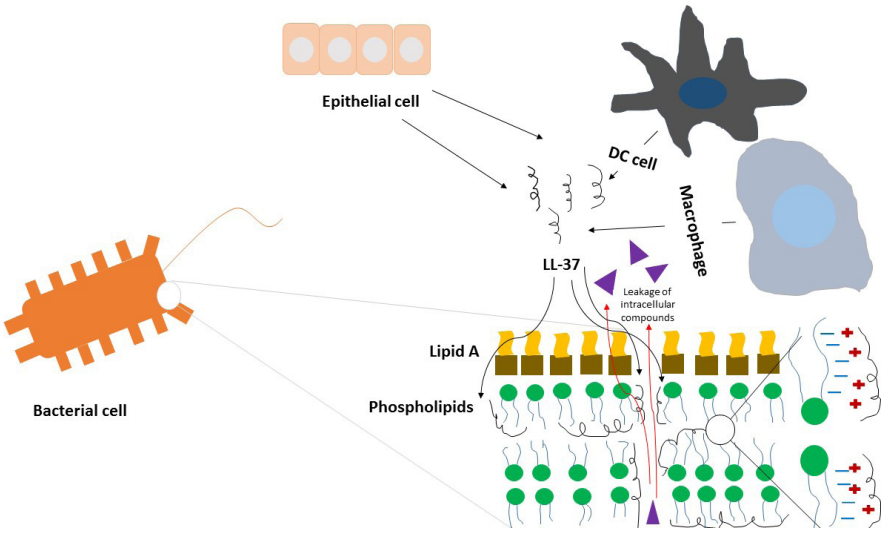

Figure 1. The Mechanism Action of LL-37 Peptide Against Gram-Negative Bacteria.

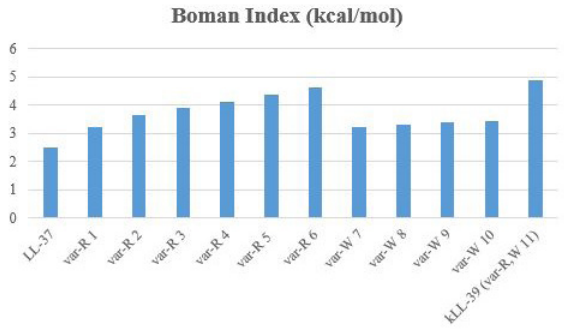

Figure 2. The distribution of Boman index in various variants of LL-37; which are R and W as arginine and tryptophan respectively.

values for all amino acids in a sequence and represents the potential of a peptide for binding to the membrane or other proteins, and values greater than 2.48 kcal/mol indicate the ability to bind to the desired peptide.

The hydrophobicity and allelometric index of the candidate analogs of LL-37 were analyzed applying the heliQuest database (http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParamsV2.py). The spiral hydrophobicity index is a scale for determining the helix amphiboletic index. The length and direction indexes of vector >µH< depend on the degree of hydrophobicity and the position of the lateral chain along the spiral axis. The amount of >µH< is 0-3.26 as well. Then, the probability of the hydrolysis of the peptides was evaluated by the Motif online application (http://crdd.osdd.net/raghava/hemopi/design.php) using the SVM + SVM1 algorithm and the PROB index comparisons. Meanwhile, the PROB index can be changed between 0-1, indicating hydrolysis (PROB = 1) and the degradation of the proposed peptide (PROB = 0). The kLL-39 analog peptides were then evaluated by the ExPASy database (https://web.expasy.org/protparam/). Binding to anionic lipids on the BCM is one of the main mechanisms of peptide LL-37 due to the positive net charge and the nature of the cationicity of this peptide. Undoubtedly, the analog kLL-39 should be similar to LL-37 peptide by binding to the BCM thus, the TMHMM online server (http://www.cbs.dtu.dk/services/TMHMM) was used to achieve this goal.

The nature of amphipathicity of the synthetic kLL-39 peptide was confirmed by drawing helical-wheel (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi), immunogenic identifying (http://imed.med.ucm. es/tools/antigenic.pl), and allergenicity (http://crdd.osdd.net/raghava/algpred/). Subsequently, the 3D spatial shape of this peptide was synthesized by I-TASSER online application (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Figures 3-5).

Finally, the synthetic peptide sequence (i.e., mLL-37) by the Predict Antimicrobial Peptides, the Predicted Antimicrobial Region within Peptides, and the Rational Design of antimicrobial Peptides contained in the CAM_{R3} online database (http://www.camp.org) was evaluated and approved in order to ensure that analog kP-39 was an AMP (http://www.camp.bicnirrh.res.in).

Results

The LL-37 sequence was obtained under the access code AP00310 from the Antimicrobial peptide database and aligned with similar antimicrobial peptides in order to compare, identify the conserve sites, and eventually, compare the spectrum.

LL-37 Analog Candidate Synthesis

A number of analogues were designed regarding the sequencing of LL-37 peptide and maintaining its functional areas. In all of these analogues, the amino acids of the serine peptide were racemized (Form D-Ser), valine amino acid was replaced by isoleucine, and two glutamine amino acids were added near the N-terminal of the peptide to reduce the possible effect of LL-37 peptide on the progression of Alzheimer disease. Then, the R and W amino acids were replaced by K and L, respectively.

According to a preliminary analysis, LL-37 peptide was detected as an amphipathic cationic peptide which contained α-helicate structure with 35% hydrophobic residues. Next, the general characteristics of LL-37 analogues were compared to LL-37 to exclude analogues peptides that lost the overall LL-37 properties. In addition, the values of the Boman index were calculated for each candidate analog peptide and listed in Table 1. Based on computer calculations, the position of R and W amino acids had no effect on Boman index increment, but in

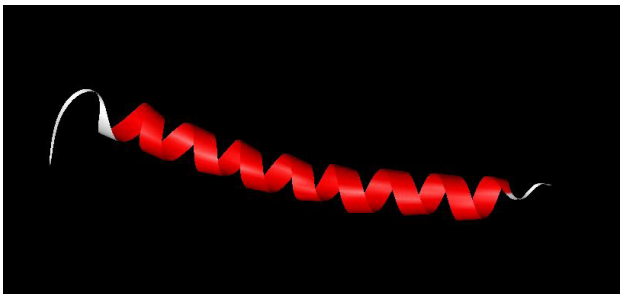

Figure 3. Tertiary structure of kLL-39 which is predicted with high rate score via I-TASSER online server.

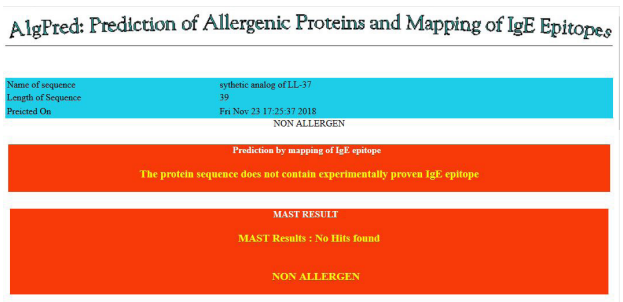

Figure 4. Prediction of potential allergenicity of kLL-39 by Alg-Pred online tool.

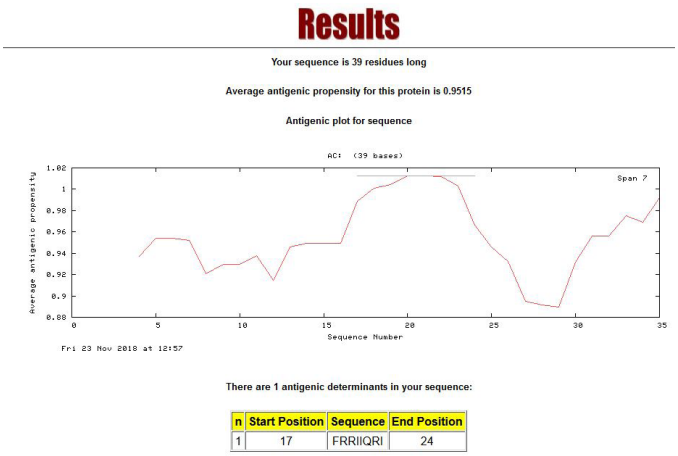

Figure 5. Computational analysis of immunogenicity domains of kLL-39.

general, an increase in the number of R and W amino acids increased Boehm index, especially the R amino acid, leading to a significant increase in the trend of peptide potential binding index to the membrane. The increase in the Boman index confirms the efficacy of an AMP.[21]

Generally, peptides that contain more hydrophobic amino acids have a better effect on binding to the membrane.[22] However, it should be noted that excessive hydrophobic uptake leads to the hemolysis of the peptide.[23] The results of the hydrophobicity and hydrophobic index of LL-37 analogues candidates are provided in Table 1.

As regards the polar nature of R and K amino acids, it was expected that the level of the hydrophobicity of the analogue R would not change significantly. Based on the average calculation, the dispersion coefficient and the standard deviation of the hydrophobic index of the analogs R and W, it was determined that the replacement of R in the peptide sequence LL-37 failed to change significantly. Conversely, the replacement of W instead of L caused a significant increase in the degree of hydrophobicity and hydrophobic index of analogue peptides. Furthermore, the position of W substitution did not affect the increase of these indexes. However, the analogue peptide kLL-39 (keikha-LL39 amino acid fragment) was identified as the most appropriate analogue of LL-37 peptide and was considered for final analysis.

Considering that increasing the hydrophobicity of an antimicrobial peptide increases its probability of hydrolysis in the host, the probability of hemolysis of the peptide was calculated by HemoPI online server.

Likewise, the characteristics of the synthetic peptide kLL-39 were examined by the ProtParam database (Table 2).

Based on the results of the online TMHMM server software analysis, it was revealed that kLL-39 failed to pass through the membrane and was located at the extracellular surface of the BCM, which reacted with it through electrostatic interactions and the creation of a pore (Figure 6).

Similarly, the helical-wheel design confirmed the amphipathic nature of k-LL-39 (Figure 7) and the kLL-39 peptide sequence analysis approved the presence of an immunogenic segment.[17-24] Moreover, considering the

Table 1. The List of LL-37 Peptide Variants With Hydrophobic Information

Peptide Name	Amino Acid Change	Hydrophobicity	
		Level H	Index μH
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	0.140	0.544
Var 1	LLGDFFRRSKEKIGKEFKRIIQRIKDFLRNLEEVPRTES	0.139	0.545
Var 2	LLGDFFRRSREKIGKEFKRIIQRIKDFLRNLEEVPRTES	0.138	0.544
Var 3	LLGDFFRRSRERIGKEFKRIIQRIKDFLRNLEEVPRTES	0.137	0.544
Var 4	LLGDFFRRSRERIGREFKRIIQRIKDFLRNLEEVPRTES	0.134	0.546
Var 5	LLGDFFRRSRERIGREFRRIIQRIKDFLRNLEEVPRTES	0.134	0.546
Var 6	LLGDFFRRSRERIGREFRRIIQRIRDFLRNLEEVPRTES	0.134	0.546
Var 7	WLGDFFRKSKEKIGKEFKRIIQRIKDFLRNLEEVPRTES	0.171	0.536
Var 8	WWGDFFRKSKEKIGKEFKRIIQRIKDFLRNLEEVPRTES	0.201	0.566
Var 9	WWGDFFRKSKEKIGKEFKRIIQRIKDFWRNLEEVPRTES	0.201	0.566
Var 10	WWGDFFRKSKEKIGKEFKRIIQRIKDFWRNWEEVPRTES	0.201	0.566
kLL-39	WWGDFFRRSRERIGKEFRRIIQRIRDFWRNWEEVPRTES	0.197	0.567

Note. R: Arginine; W: Tryptophan and R; W: Arginine-Tryptophan.

Table 2. Physico-chemical Features of kLL-39 Peptide by ProtParam

Name	Sequence Length	Molecular Weight	pH Isoelectric	Total Negatively Charge	Total Positively Charge	Half-life	Aliphatic Index	Gravy
kLL-39	39	5197.86	11.41	7	11	2.8 hours	47.44	-1.41

results of two MAST servers and the prediction by the mapping of Immunoglobulin E, the epitope demonstrated that the synthetic peptide kLL-39 was not allergen to humans.

In order to predict the three-dimensional structure, a synthetic peptide sequence was uploaded to I-TASSER site. According to the best proposed model (C = -1.09), the kLL-39 peptide had an alpha-helicoidal structure similar to antimicrobial peptide LL-37 (Figure 3).

In the final assessment, the antimicrobial properties of the kPL-39 synthetic peptide were evaluated by the online CAMPR3 database. Based on the bioinformatics analysis, it was found that kLL-39 peptide was an antimicrobial peptide with 97% consideration.

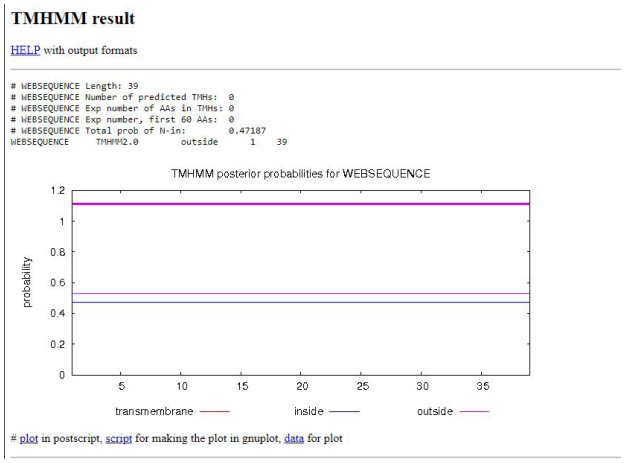

Figure 6. Prediction of extra-cellular localization of kLL-39 candidate peptide via TMHMM server.

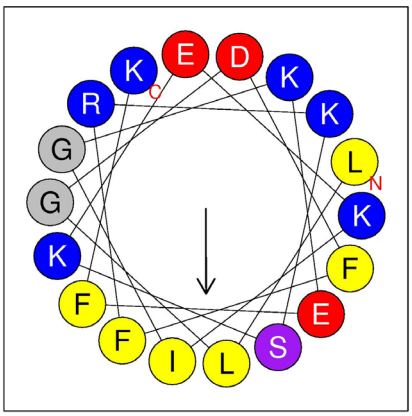

Figure 7. The helical-wheel stricter of kLL-37 peptide using HeliQuest server.

Discussion

Today, due to the emergence and spread of resistant infections worldwide, new alternatives such as AMPs are needed in the treatment and control of infections. Merrifield et al[24] and Spitznagel[25] developed the historically use of small peptides as anti-infectants. The LL-37 peptide is one of the best examples of antimicrobial peptides. In other words, it is the α-helicate with a human source that has a wide spectrum activity against pathogen microorganisms. According to reports, LL-37 has an acceptable inhibitory effect against *S. aureus*, Streptococcus group A, B, *Enterococcus faecalis*, *E. coli*, *P. aeruginosa*, *Salmonella typhimurium*, MDR *A. baumannii*, and *Candida albicans* as well. In addition, LL-37 induces immune-cytokine production, wound healing, chemotaxis agent, and angiogenesis.[2,3,26,27]

In spite of the above-mentioned information, antimicrobial peptide production encounters many challenges. For example, the significant challenges to the AMPs include the high cost of production, inefficient-bioavailability, the lack of volunteers in clinical trials, the reports of resistant pathogenic bacteria (e.g., *Burkholderia* spp), the modifications of the bacterial membrane, AMPs extracting efflux pumps, and the suppression of AMPs expression.[15,28,29]

Computer and bioinformatics analyses are considered as one of the most essential methods for reducing the costs of new AMPs design and production. In other words, using the computer analysis, you can make some small changes on the modifiable AMPs that are more effective and non-toxic for the host cells.[2,30] In general, three major methods are proposed for bioinformatics design of antimicrobial peptides, including template-based studies, biophysical studies, and virtual screening. Template-base designs are one of the most reliable methods that create some modifications in one AMP and produce one more effective synthetic peptide, which enhances the microbial properties while not having any toxicity for the host. AMPs such as cecropin, magainin, protegrin, or lactoferricin are among the most important examples of Temple-based AMPs.[2]

It is found that LL-37 might be involved in the progression of Alzheimer disease.[12-14,31] Therefore, in the present study, we attempted to remove the suspicious structures from LL-37 peptide sequence. Furthermore, previous studies showed that tryptophan residue increases the stability of peptides and has an active role in enhancing the inhibitory effect of bacterial growth.[18] Meanwhile, the creation of amphipathic helicoidally structures by using

arginine and valine residues leads to an increase in the rate of microbial activity and the specificity of the binding of the peptide to the bacterial membrane.[32]

In the present study, we attempted to produce a new antimicrobial peptide with higher microbial activity and less toxicity to the host by replacing the arginine and tryptophan amino acids instead of lysine and leucine. According to computerized calculations, the kLL-37 synthetic peptide with a 97% confidence can be a new antimicrobial peptide, but it requires clinical designing, implementation, and investigation for final confirmation.

Ethical Approval

Mashhad University of Medical Sciences approved this study.

Conflict of Interest Disclosures

None.

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