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Design and Mode of Action of a New Synthetic Antibacterial Peptide against *Pseudomonas aeruginosa*

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ABSTRACT

Background: Research into antimicrobial peptides (AMPs) and other novel antimicrobial strategies continues to be a focus of the scientific community to address the growing challenges posed by antibiotic resistance. The aim of this study was the specific bioinformatic design of a novel hybrid AMP that combines the potent antibacterial properties of Temporins and Brevinin while minimizing its cytotoxic effects.

Methods: The N-terminal of Brevinin peptides and the C-terminal of Temporin peptide were selected. To optimize the peptide, we replaced the amino acids at positions 1, 3, and 7 with glycine, lysine, and lysine, respectively. Various approaches were used to assess physicochemical characteristics, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), cytotoxicity, apoptosis, hemolytic activity, and reactive oxygen species generation.

Results: The novel peptide had 57% hydrophobicity and four positive electric charges due to its lysine amino acid composition. The synthesized peptide exhibited MIC and MBC values of 80 µM against *Pseudomonas aeruginosa*. According to the MTT assay, the peptide showed neither cytotoxic nor hemolytic effects on human cells. The results of electron microscopy showed that this peptide can disrupt the integrity of *P. aeruginosa* membrane cells and induce morphological changes in their surface structure.

Conclusion: This research suggests that the synthetic peptide could serve as a promising potential therapeutic agent to combat antibiotic-resistant strains of *P. aeruginosa*. ***DOI: 10.22034/IBJ.30.1.81***

Keywords: Pseudomonas aeruginosa, Synthetic peptide, Mode of action

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1. INTRODUCTION

The use of antibiotics has reduced the incidence of bacterial infections and human mortality; however, the frequent use of these drugs not only causes bacterial resistance but also leads to adverse disturbances in microbiota, which plays an important role in various functions in human biology[1,2]. During the last 30 years, only two new classes of antibiotics have been introduced to the market. Therefore, with the increase in antibiotic resistance, there is an urgent need for new treatments for infections[3].

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen with high levels of innate and acquired antibiotic resistance. It is a common cause of healthcare-associated infections such as pneumonia, bloodstream, urinary tract, and surgical site infections[4]. Due to the characteristics of *P. aeruginosa,* such as limited permeability in the outer membrane, acquiring mutations in the efflux pump, encoding an inducible AmpC beta-lactamase, and carrying plasmids with

resistance elements, there is a need to use second- or third-line drugs[5].

Antimicrobial peptides (AMPs) provide opportunities for addressing the aforementioned challenges, leading to increased interest in the identification and optimization of these peptides[6]. AMPs are a class of antibiotics that form part of the innate immune system in many organisms. Polymyxins, especially colistin, were originally derived from bacteria and considered the first AMPs used in therapy. These peptides are small molecules (10-50 amino acids) that are amphipathic, polycationic, and structurally diverse. They have a variety of biological activities, including antibacterial, antiviral, antifungal, antiparasitic, anticancer, and immune-regulatory functions. Other advantages of AMPs include high solubility, low cytotoxicity in eukaryotic cells, resistance to mutation, low molecular weight, and strong thermal stability[7,8]. AMPs are a promising alternative to traditional antibiotics due to their activity against a broad spectrum of microbes and mode of action, which is distinct from those used by current antibiotics. Also, AMPs cause bacterial death through initial electrostatic interactions with the lipid bilayer. This action disrupts membrane structure and forms transient pores, increasing the permeability of microbial membranes[9]. Therefore, AMPs are considered potential antimicrobial therapeutic agents because peptides targeting the bacterial membrane are less likely to induce bacterial resistance, as multifactorial resistance mechanisms are required in the cell membrane[10]. Although AMPs have been proven to be effective, they are non-specific and can target a wide range of Gram-negative and Gram-positive bacteria. Therefore, the search for new AMPs has become an important area of study, and to be clinically useful, they must show high selectivity for bacterial membranes with low toxicity for mammalian cell membranes[11].

Temporins and Brevinins are two main families of AMPs found in amphibians, playing an important role in their innate defense systems[12]. Functional studies have shown that despite their small size and low net positive charge, Temporins can target Gram-positive and Gram-negative bacteria, fungi, protozoa, viruses, and red blood cells. However, their effects on red blood cells can lead to toxicity in humans, limiting their clinical use[13,14]. Brevinins are a superfamily of linear, amphipathic, and cationic peptides that show strong antibacterial, antifungal, and antiviral activities. Unfortunately, most Brevinins have strong hemolytic properties that reduce their application as antimicrobial agents[15].

Given that Tempurin and Brevinin are both potent natural antibacterial peptides but exhibit toxicity to red blood cells, this study aimed to bioinformatically design a novel hybrid AMP that maintains the antibacterial properties of both families of AMPS while minimizing cytotoxic effects. The design strategy has been focused on combining conserved fragments from both Brevinin and Temporin peptide families to produce a new hybrid peptide with unchanged antibacterial activity and reduced toxicity. The hybrid peptide was successfully synthesized, and then its antibacterial effect against *P. aeruginosa* as well as its toxicity on a mammalian cell line were evaluated.

2. MATERIALS AND METHODS

2.1. Peptide design and synthesis

Temporins and the Brevinins were selected from the APD3 website (https://aps.unmc.edu) to design the new peptide. The same regions in each isotype of these peptides were examined, and the FLSAIAS sequence from the N-terminal of Brevinins peptides and the NLAKKIL sequence from the C-terminal of Temporins peptides were selected. As a result, the amino and the carboxyl ends of the new peptide were placed as FLSAIASNLAKKIL. To optimize the peptide, we replaced the amino acids at positions 1, 3, and 7 with glycine, lysine, and lysine, respectively, which obtained a new sequence of GLKAIAKNLAKKIL, for further study.

2.2. Physicochemical properties and structure prediction

The ProtPARAM tool on the EXPASY site (https://web.expasy.org/protparam/) and the ADP3 site were used to check molecular weight, isoelectric pH, number of charged amino acids, peptide charge type, peptide half-life, peptide stability, aliphatic index, and the grand average hydropathy (GRAVY) score. Also, the GOR IV server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl? page=/NPSA/npsa_gor4.html) and the pep-fold server (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD) were used to predict the secondary and tertiary structure of the peptide, respectively. Finally, after repeated checks, the desired peptide was synthesized by GenScript Company, USA.

2.3 Peptide synthesis

The peptide was chemically synthesized by using N-(9-fluorenyl) methoxycarbonyl (Fmoc) according to the standard solid-phase methods[16]. The purity of the synthetic peptide was confirmed by the reverse-phase HPLC, with a purity of >90%. Its molecular weight was assessed by electrospray ionization mass spectrometry.

2.4. Analysis of secondary structure of the synthetic peptide

The ultraviolet circular dichroism (CD) spectrum of peptides can determine important features of the

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secondary structure, such as the content of alpha helix, beta sheet, or some other conformations presented. Thus, CD was used to analyze the secondary structure of the peptide. The purified peptide solution (0.2-0.5 mg/mL) in 70% trifluoroacetic acid (TFE) was loaded into a 1 mm quartz cell, and its spectrum was scanned from 190 to 250 nm using a spectrophotometer Jasco J810 (Jasco, Japan) at 25°C with a scan speed of 200 nm/min.

2.5. Antimicrobial activity assays

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were evaluated to determine the antimicrobial activity of the peptide[17]. MICs are defined as the lowest concentrations of antimicrobial drugs that inhibit the visible growth of microorganisms following overnight incubation. After preparing a bacterial suspension, MICs and MBCs were used to determine the antimicrobial activity of the synthetic peptide[17]. A bacterial suspension adjusted to a 0.5 McFarland standard (approximately 5×10^5 CFU/mL of *P. aeruginosa* ATCC 27853) was prepared. Then, 100 µL of the bacterial suspension and 100 µL of the peptide at concentrations ranging from 200 to 1 µM were incubated at 37°C for 16 hours. The sterilized Mueller-Hinton broth (containing the microorganism cultured in the medium without the peptide) was used as a negative control, and meropenem (a commercial antibiotic at a concentration of 4 mg/L) was used as a positive control. After 16 hours of incubation, the turbidity in each well was checked, and the results were read using an ELISA reader. For the MBC assay, 100 µL of the contents of each well showing no visible growth was plated onto a Mueller-Hinton agar plate (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C for 24 h. MBC results were defined as the lowest concentration of bactericidal peptide. Each experiment was performed in triplicate.

2.6. Cytotoxicity assay

The cytotoxic effect of the synthetic peptide on the human embryonic kidney (HEK293; Pasteur Institute of Iran, Tehran). The cell line was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) method[18]. Briefly, 8,000 cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin/streptomycin under 5% CO_2 at 37°C. After 24 h, the culture medium was changed, and cells were treated with ½, 1, and 2 MIC concentrations of peptide for 24 h. Then, 20 µL of MTT dye (5 mg/mL) was added to each well and incubated at 37°C for 4 hours. In the end, the culture medium was removed, 100 µL of dimethyl sulfoxide was added to each well, and the OD of each well was read at a wavelength of 570 nm using an ELISA reader. PBS buffer and 1% Triton X-100 were used as negative and positive controls. Each experiment was performed in triplicate.

2.7. Hemolytic activity

The hemolytic activity of the synthesized peptide was tested according to a previous method with slight modifications[18]. Briefly, red blood cells were collected and then washed with PBS to remove all broken red blood cells. Then, 2% cell suspension from RBC was prepared using PBS. Also, ½, 1 and 2 MIC concentrations of peptide were mixed with red blood cells in microcentrifuge tubes. After 2 hours of incubation at 37°C, the tubes were centrifuged, and the suspension was transferred to a clear 96-well plate. The absorption of hemoglobin released in the suspension was detected by a plate reader at a wavelength of 567 nm. In addition, red blood cells treated with PBS and 1% Triton 100-X were used as negative and positive controls, respectively. Each experiment was performed in triplicate.

2.8. Evaluation of bacterial cell membrane integrity

Evaluation of bacterial cell membrane integrity after being exposed to the synthetic peptide was performed using propidium iodide (PI) staining with modifications[19]. A bacterial suspension adjusted to 0.5 McFarland concentration was treated with the 1 and 2 MIC concentrations of synthetic peptides and incubated at 37 °C for 4 hours. The supernatant in each well was carefully removed, and the bacterial cells were washed with PBS. Then, bacterial cells were stained with PI solution with a final concentration of 50 µg/mL in PBS in a dark place at room temperature for 20 min. Next, the cells were harvested by centrifugation, washed, and resuspended in PBS. The percentage of PI-positive cell bacteria was determined using a FACS flow cytometer (BD BD-FACSCalibur, BD Biosciences, USA). The untreated cells were used as a negative control.

2.9. Measurement of reactive oxygen species (ROS) production

The ROS production in *P. aeruginosa* cells was evaluated using the fluorescent dye 2',7'-diacetyldichlorofluorescein (DCFH-DA) following the manufacturer's instructions[20]. Following the preparation of the bacterial suspension, it was treated with a concentration of 0.5 McFarland and 1 and 2 MIC of the peptides and incubated at 37°C for 4 hours. After incubation time, the cells were washed by adding 2 mL of PBS solution. To measure intracellular ROS in bacterial cells, we incubated the cells with DCFH-DA for 45 minutes and then analyzed them using a flow cytometer (Becton-Dickinson, USA).

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2.10. Evaluation of bacterial cell structure by electron microscopy

Electron microscopy is suitable for examining surface and internal structures at micron and nanometer dimensions, though the examined samples require special preparation. After preparing a bacterial suspension at half the McFarland standard (0.5 McFarland), 2× MIC of the peptide was added to 500 µL of this 0.5 McFarland suspension and incubated for 4 hours. Next, 500 µL of the 0.5 McFarland suspension was used as a negative control. After incubation, the samples were centrifuged at 5000 ×g for 5 minutes, the supernatant was discarded, and the pellet was washed with PBS buffer. The centrifugation and supernatant removal were repeated. The remaining pellet was placed on a slide to be dried and then fixed with 4% and 8% formaldehyde. The samples were washed three times with PBS, and after draining the buffer, alcohol was used to dehydrate the samples on the slides. Finally, the slides were dried at room temperature. The prepared samples were examined using a scanning electron microscope. According to the type of test, imaging of the samples was performed using BSE and SE detectors at different magnifications with an electron microscope (FEI ESEM QUANTA 200, USA), and elemental analysis of different phases observed in the images was conducted using a detector (EDAX EDS Silicon Drift 2017, USA).

2.11. Statistical analysis

All the statistical analyses were performed using GraphPad Prism version 8.01 (GraphPad Software, San Diego, CA, USA) using one-way ANOVA. The group treated with the peptide was compared to the untreated (control) group, and the results were expressed as mean ± standard deviation. The $p \leq 0.05$ was considered statistically significant.

3. RESULTS

3.1. Design and prediction of antimicrobial properties of peptides

The antibacterial properties of the GLKAIAKNLAKKIL sequence were determined using the APD3 software. This peptide contained four lysine amino acids, resulting in a total of four positive charges and a molecular weight of 1480,9 Da. Additionally, the peptide was composed of 57% hydrophobic residues, and its Boman index was -0.15 kcal/mol. The physicochemical properties of this peptide included a pH isoelectric of 10.48, GRAVY score of 0.45, and aliphatic index of 160.71. The estimated half-life of this peptide was 30 hours in mammalian reticulocytes and laboratory conditions, more than 20 hours in yeast and animal models, and more than 10 hours in *Escherichia coli* bacterium. The instability index calculated for the new peptide was 10.91, which is classified as a stable peptide. The GOR4 server was used to analyze the secondary structure of the peptide, which consisted of 28.57% alpha helix, 42.86% random coil, and 28.57% extended strand (data not shown). Prediction results from the PEP-FOLD3 server indicated that the peptide adopted an alpha helix conformation (Fig. 1).

3.2. Peptide synthesis

The peptide was chemically synthesized and purified to >90%. The purity of the synthesized peptide was confirmed by RP-HPLC and mass spectroscopy.

3.3. Analysis of secondary structure of the synthetic peptide

CD is a spectroscopic method used to analyze the structure of biomolecules in solution. It is particularly valuable for determining the link between structure and function in biomolecules, such as proteins. CD allows for precise monitoring of structural changes influenced by the surrounding environment. In particular, this technique enables the detailed examination of the secondary microstructure, as well as the changes occurring in the superstructure of biomolecules such as proteins. The data derived from CD analysis revealed that the secondary structure of the peptide, when dissolved in a water buffer, consisted of 28.57% alpha helix, 42.86% random coil, and 28.57% extended strand (Fig. 2).

3.4. MIC and MBC results

The MIC test was conducted using the broth microdilution method to evaluate the effectiveness of the novel peptide against the reference strain of *P. aeruginosa* ATCC27853. The MIC and MBC concentrations of the synthesized peptide against *P. aeruginosa* were both found to be 80 µM.

Fig. 1. Peptide structure prediction by the PEP-FOLD3 server, indicating that the synthetic peptide adopts an alpha helix conformation.

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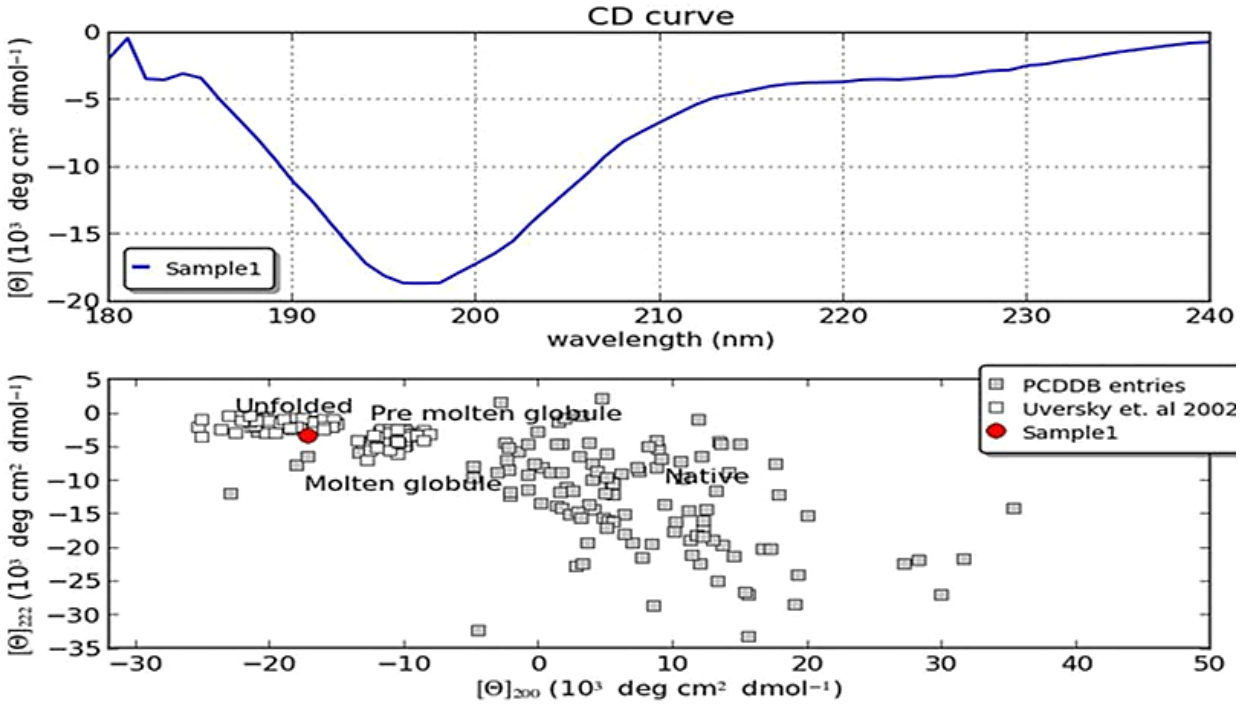

Fig. 2 CD spectra analysis showing that the peptide in 70% TFE solution has a mixed secondary structure composed of 28.57% alpha helix, 42.86% random coil, and 28.57% extended strand.

3.5. Cytotoxicity and hemolytic activity

The MTT results indicated that the peptide did not exhibit any cytotoxicity towards human cells at concentrations of ½ MIC, MIC, and 2 MIC, as compared to the negative control group (data not shown). Also, the blood hemolysis rates of the peptide at 2 MIC, MIC, and 1/2 MIC concentrations were 6.57%, 2.86%, and 2.58%, respectively. These rates did not differ significantly from those of the negative control group. Overall, the findings indicate that the designed peptide lacks hemolytic properties.

3.6. Bacterial cell membrane integrity

In this study, the membrane integrity of *P. aeruginosa* cells was evaluated after treatment with 1 and 2 MIC concentrations of synthetic peptide via monitoring the PI uptake. PI is a DNA-staining dye that tightly binds to nucleic acids and gives a red fluorescence. It can penetrate the cells only if the integrity of the plasma membrane is disrupted. The percentage of PI-positive *P. aeruginosa* cells increased from 8.97% (in the negative control) to 39.3% (at MIC level) and to 71% (at 2 MIC level), indicating that the number of dead cells increased with increasing concentrations of the peptide treatment (Fig. 3A-3C). These results indicate a statistically significant increase in cell death after peptide treatment compared to the control group ($p \leq 0.05$).

3.7. ROS production

The ROS assay evaluated the level of free radical generation in *P. aeruginosa* bacteria upon exposure to the peptide. This experiment aimed to compare the impact of the peptide on bacterial free radical formation in relation to the negative control. The mean fluorescence intensity (MFI) values represent the color intensity in each sample. The color intensity in the negative control was significantly lower than that of the bacteria treated with the peptide at the MIC and 2 MIC ($p \leq 0.05$). The MFI values were 20.3 for the negative control, 94.4 for bacteria treated with the peptide at MIC, and 172 for bacteria treated with the peptide at 2 MIC, indicating a dose-dependent increase in ROS production (Fig. 3E-3G).

3.8. Electron microscopy of peptide-treated bacterial cells

The results of the electron microscopy indicated that the peptide was effective against the membrane of *P. aeruginosa* and destroyed this bacterium. The untreated bacterium showed a smooth surface, while those treated with the peptide displayed a wrinkled appearance (Fig. 4). These results showed that the peptide can disrupt the integrity of *P. aeruginosa* cells and induce morphological changes in their surface structure.

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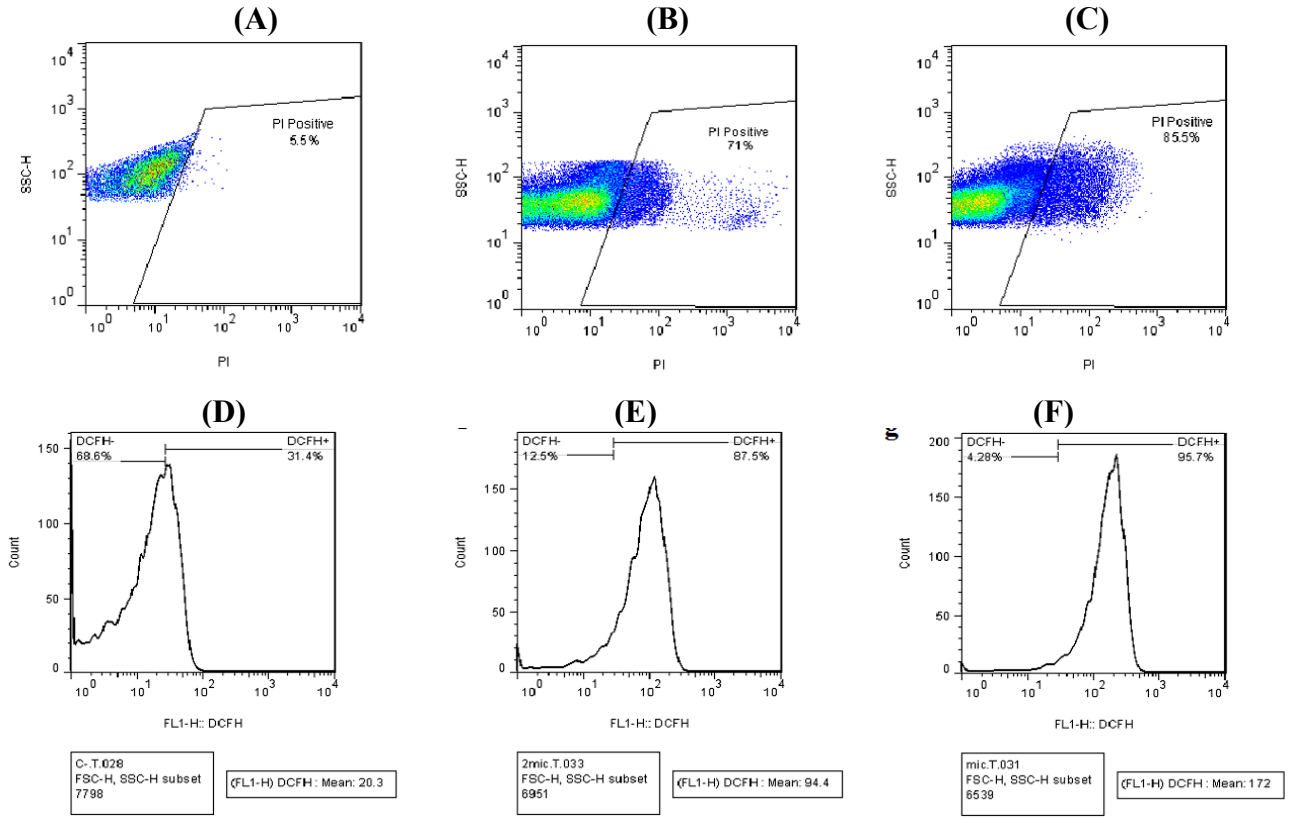

Fig. 3. (A-C) Evaluation of bacterial cell membrane integrity using PI uptake. The side-scatter (SSC) and forward-scatter (FSC) plots were used to determine the analysis boundary relative to untreated cells (control). (A) negative control, (B) MIC, and (C) 2 MIC concentrations of the peptide, respectively. (D-F) The mean MFI of the color produced by the BD FACSCalibur. The level of DCFH expression on the right side of the diagram is specified in the upper part, and below each diagram, a number reported as MFI indicates the color intensity in each sample[21]. (D) negative control, (E) MIC, (F) 2 MIC concentration of the peptide, respectively.

4. DISCUSSION

The current increase in multidrug-resistant bacteria is a global health concern. This crisis highlights the urgent need to design and develop new classes of antimicrobial molecules. Recent advances have led to the emergence of AMPs as a viable antibacterial strategy, due to their broad-spectrum activity, potent effectiveness on Gram-negative bacteria, and infrequent occurrence of drug resistance[9,22]. However, despite many desirable properties, AMPs may also exhibit undesirable properties such as cytotoxicity or hemolytic effects, reduced antimicrobial activity in the presence of salt, and high cost of production[23,24]. Nonetheless, these obstacles can be overcome through various approaches, especially by modifying peptide sequences. To create AMPs with improved characteristics, many studies have found that truncation and amino acid substitution can be considered as effective strategies[25,26]. Hybrid peptide derivatives, which are combinations of two or more native peptides, have attracted a lot of interest because they can improve the antimicrobial effects of their parent peptides. Modifications have been made to increase the activity of the AMPs and reduce the cytotoxicity of natural AMPs, resulting in consistently smaller sizes and increased cationic properties[23].

In this study, the conserved sequences of two peptide families, Temporin and Brevinin, were selected and analyzed. By combining the NLAKKIL sequence from the Temporin peptide with the FLSAIAS sequence from the Brevinin peptide, we created a novel peptide, FLSAIASNLAKKIL. To further optimize the amino acid sequence, we replaced the residues at positions 1, 3, and 7 with glycine, lysine, and lysine, respectively, resulting in the new sequence GLKAIAKNLAKKIL.

A 2015 study revealed that the novel hybrid T9W peptide showed strong activity against *P. aeruginosa*, including strains resistant to ceftazidime, gentamicin, and ciprofloxacin, and with efficacy comparable to several existing anti-Pseudomonas agents. T9W showed strong activity against *P. aeruginosa* and weak activity

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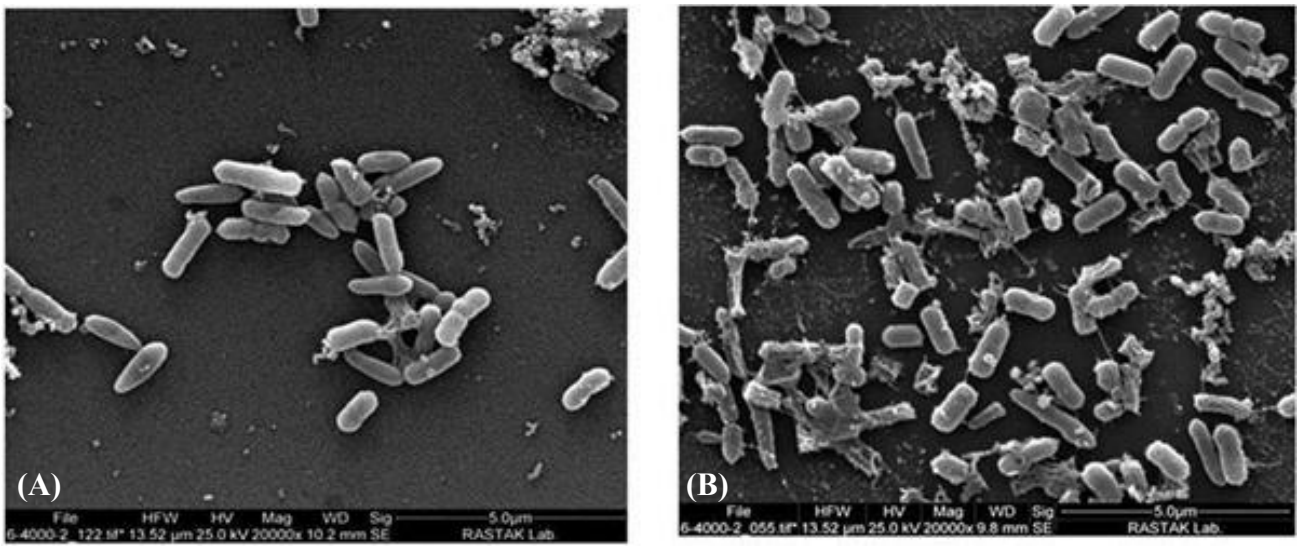

Fig. 4. Electron microscope view (A) without peptide and (B) with peptide at 2 MIC concentration on *P. aeruginosa.* The bacterium treated with the peptide has a wrinkled surface.

against other Gram-negative and Gram-positive bacteria. Importantly, T9W showed no hemolytic activity against human red blood cells at concentrations up to 256 µM in vitro[26]. Also, research by Klubthawee et al. has demonstrated that the hybrid AMP PA-13 exhibited remarkable broad-spectrum antibacterial activity, particularly against *P. aeruginosa*, with no toxicity toward human red blood cells and L929 cells. Similar to the method used in our study, PA-13 was designed based on the conserved sequences of 45 α-helical cathelicidins and 11 α-helical aureins[23].

One disadvantage of AMPs is their potential toxicity to eukaryotic cells. Therefore, designing hybrid peptides can help reduce toxicity compared to their parent peptides. Our investigation revealed that the AMP we synthesized did not exhibit any harmful effects on human cells at concentrations of 2 MIC, as compared to the negative control group. The toxicity and antibacterial activity of AMPs are affected by several physicochemical properties, such as length, charge, hydrophobicity, amphipathicity, and hydrophobic/hydrophilic angle. Consequently, the alteration of one component may lead to variations in the others, making it difficult to separate the influence of a single element on the overall activity[27]. Another advantage of our synthetic peptide is the identical MIC and MBC values, which indicate its strong bactericidal activity, as the concentration needed to inhibit bacterial growth is the same as that required to kill the bacteria. This outcome may also be influenced by factors such as the bacterial strain tested, peptide stability, and assay sensitivity. Multiple studies have reported that MIC and MBC values are identical for some AMPs[28,29]. For example, research by Yasir et al. demonstrated that the Mel4 peptide exhibited identical MIC and MBC values against *P. aeruginosa* ATCC 19660[28].

As mentioned above, the fluorescent dye PI can enter bacterial cytosol only when membrane integrity is lost. Findings obtained from PI uptake studies revealed that our peptide likely induces bacterial cell death by disrupting the integrity of the bacterial membrane.

Results from measuring ROS production in *P. aeruginosa* suggested that our synthetic peptide could induce a significant elevation in ROS levels within bacterial cells. As noted by Vaishampayan and Grohmann, ROS interacts with multiple cellular targets, including proteins, lipids, and nucleic acids, which can lead to oxidative stress in bacteria and is considered an initial factor in apoptosis[30]. Similarly, Ning et al. showed that ROS levels in Staphylococcus aureus cells treated with glycinin basic peptide increased significantly in a concentration-dependent manner[20].

A previous study has shown that AMPs have the ability to bind to negatively charged bacterial membranes and/or lipopolysaccharides[31] through electrostatic interactions, which can be enhanced by imparting a positive charge to the AMPs[32]. Our results from electron microscopy indicated that the peptide we designed was effective in targeting the membranes of the *P. aeruginosa* bacterium. Taken together, in vitro characterization of AMPs could be useful in predicting the safety and efficacy of these compounds in vivo. Based on our in vitro findings, we can also predict the safety and effectiveness of our synthesized peptide. To advance the clinical development of this peptide, further investigations should be carried out to assess its efficacy and safety in animal models.

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5. CONCLUSION

In this study, a novel peptide was created and synthesized based on the conserved fragments from two-peptide families, Temporin and Brevinin, with several amino acid substitutions. We found that this peptide induced wrinkles in the bacterial membrane and disrupted the integrity of *P. aeruginosa* cells, leading to morphological changes on the cell surface. The low levels of cytotoxic and hemolytic activity of the peptide against mammalian cells indicate its therapeutic potential. Our results provide further evidence that rational hybrid peptide development can be an effective strategy for developing new antibacterial medicines.

DECLARATION

Acknowledgments

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Generative AI and AI-assisted technologies

In this manuscript, AI tools (ChatGPT, DeepSeek) were used exclusively to enhance the clarity, grammar, and overall readability of the text. All scientific content, figures, and conclusions were generated by the authors.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

AA: data curation, formal analysis, methodology, writing–original draft; HMH: supervision, conceptualization, investigation, formal analysis, methodology, validation, visualization, writing–review & editing; SAM: data curation, formal analysis; SK: data analysis, writing–review & editing; JA: project administration, validation, conceptualization.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version does not contain supplementary material.

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