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Original Article

Activity of a Novel Antimicrobial Peptide with Nitric Oxide Induction against some Pathogenic Bacteria

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Abstract

Introduction: Bacterial resistance against antibiotics has caused many problems in treating humans and animal infections worldwide. Nowadays, researchers are continuously seeking to develop novel antibacterial to tackle the issue of microbial resistance. Antimicrobial peptides have been introduced as new effective strategies that kill bacteria quickly and cause less antibiotic resistance. In this study, we evaluated the antibacterial and cytotoxic effects of a synthesized peptide (NRWCFAGRR-NH_2) on some Gram-positive and –negative bacteria and eukaryotic cells.

Materials and Methods: Twelve bacterial strains were selected to study the antimicrobial effect of the NRWC peptide. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays were used to study the bacteriostatic and bactericidal activity of these peptides, respectively. The cytotoxic effect of the peptide was evaluated on Hela cell line and human RBC using the MTT assay and hemoglobin release measurement, respectively. The J774 macrophage cell line was used to measure nitric oxide production in response to the peptide.

Results: The results showed that NRWCFAGRR peptide has a bactericidal and inhibitory effect on all 12 bacterial strains' growth in a dose-dependent manner. It has also been proven that the toxic effect of the peptide on human cells is evitable at the MIC and MBC concentration. The highest amount of nitric oxide production was induced after 48 hours of treatment.

Conclusions: Considering the research conducted in the field of antimicrobial peptides, our designed peptide has antimicrobial properties that kill some of the pathogenic microorganisms directly and can theoretically kill some organisms indirectly via induction of nitric oxide by macrophages.

Keywords: Antibiotics Resistance, Cytotoxicity, MBC, MIC, Nitric Oxide
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Introduction

Nowadays, microbial resistance against a variety of antibiotics is considered as a global crisis. In recent years, studies on this problem have increased, and the number of research that seek to solve the problem of antibiotic resistance by peptides has been on the rise.[1] The natural antibacterial molecules that exist in animals and plants are rich resources of antibiotics like antimicrobial peptides (AMPs) are effective innate immunity and could be the first and most important choice in the new research area.[2,3] Antimicrobial peptides as part of the innate immune response have been found among all classes of life which could be used as antibiotics in bacterial infection.[4] Antimicrobial peptides are usually small peptides that are easily accessible. Unique features such as broad-spectrum activity, low toxicity, and fewer side effects have turned them into an alternative drug for antibiotics and anti-cancer drugs.[5,6]

There are several structural differences between prokaryotic and eukaryotic cells that make them a specific target of antimicrobial peptides. Antimicrobial peptides have been demonstrated to kill Gram-negative and Gram-positive bacteria, enveloped viruses, fungi, and even transformed or cancerous cells.[7] In general, these molecules, which are recognized as potent antibiotics with broad-spectrum activity, might be the right candidate in future treatment strategies.[6]

Thermostability (100 °C for 15 minutes), strong cationic properties (pI = 8.9-10.7), and fewer side effects in comparison with the common antibiotics (such as autoimmunity, resistance, toxicity, etc.) have led to more attention to these peptides for therapeutic purposes.[8]

The antimicrobial peptide's mechanism of action is usually via bacterial membrane disturbance. Since AMP has an amphipathic conformation so it is capable of reacting with both the hydrophobic and hydrophilic side of the cell membrane. In this process, positively charged peptide attraction occurs due to the negative charge of the cell membrane, and eventually, peptides penetrate the cytoplasm and disrupt the

vital operations.[9] Interaction between peptide and phospholipid has many different results like the disruption of bilayer integrity, collapse of the transmembrane electrochemical gradients, and pore formation. Despite the first natural resources of AMPs, peptide's function and stability could be improved by the introduction of different residues and chemical changes of amino acids.[10] Natural antimicrobial peptides are often cationic resulting from multiple arginine and lysine residues, and this net positive charge is extremely important for electrostatic interaction between AMPs and negatively charged bacterial membranes.[11]

Several studies increased AMP's positive charge based on the structure-function relationships by replacing neutral and acidic amino acids with cationic residues (arginine and lysine) to improve AMP's activity. In spite of their identical charge, arginine residues are more prevalent in natural antimicrobial peptides than lysine, suggesting that the guanidinium group found in arginine may be preferable for function in comparison with the amine group in lysine.[12] In this regard, we designed a peptide based on NRWCFAGDD claimed as antimicrobial peptide that inhibited *Haemophilus parasuis* in previous studies,[13] and in order to increase its net charge, both of peptide's glutamate substituted with arginine. The aim of this study was the evaluation of NRWCFAGRR peptide antibacterial effect on standard isolates of some Gram-positive and –negative pathogenic bacteria and also its cytotoxicity on eukaryotic cell lines and reticulocytes.

Materials and Methods

Cell Lines and Bacterial Strains

Salmonella enteritidis (ATCC 13076), *Bacillus cereus* (ATCC 14579), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC BAA-1705), *Serratia marcescens* (ATCC 14756), *Acinetobacter baumannii* (ATCC 19606), *Proteus vulgaris* (ATCC 8427), *Vibrio cholerae* (ATCC 39315), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Streptococcus epidermidis* (ATCC 14990) and *Nocardia brasiliensis* (ATCC 19247) were purchased (Pasture Institute, Tehran, Iran). All bacteria strains were confirmed with standard microbial laboratory tests cultured in suitable media and suspended in normal saline and the opacity adjusted to McFarland 0.5. Macrophage J774 (ATCC TIB-67) and Hela (ATCC CCL-2) cell lines were also obtained from the National Cell Bank, Pasture Institute, Tehran, Iran.

In Silico Analysis

The primary sequence analysis and physicochemical properties prediction of the peptide was made online using ProtParam (http://www.expasy.org/tools/protparam.html) and the other parameters, including Hydrophobicity, net charge PI, were calculated using Antimicrobial Peptide Database (http://aps.unmc.edu/AP/prediction/prediction_main.php). The tertiary structures of the peptide were predicted online by the PEPstrMOD server (http://osddlinux.osdd.net/raghava/pepstrmod/comb_ds.php). *In silico* prediction of the Therapeutic Index (TI) and hemolytic potency of peptides were performed using Hemopi (http://crdd.osdd.net/raghava/hemopi/) and deserv1 server (http://split4.pmfst.hr/split/dserv1), respectively.[14]

Molecular dynamics simulation makes it possible to study the behavior of peptides qualitatively at the molecular scale and to obtain a deeper analysis of various physical phenomena. The study of peptides on a molecular scale has provided the knowledge of peptide design in various applications by revealing the behavior of peptide molecules and amino acids, including their arrangement with each other, how to establish interactions and knowledge of molecular mechanisms.

In this research, a crystal structure with PDB identification code from protein database was prepared. This file contained information about the position of the protein atoms and contained the complete sequence of any existing file. Three versions of this structure were used separately to simulate at three temperatures of 27, 37 and 47 °C. The three structures were placed separately in the center of a box measuring 0.6 nm 15 15.12 × 5 / rectangular in size 31. The boxes were then filled with 12447 water molecules (model as a solvent so that a layer of water (SPC/E) about 1 nm thick surrounded the protein). Simulations were done using the GROMACS 5.1.2 software. The GROMACS 43a constant was performed using a force field of 1 atmospheric force, duration 10 nanoseconds. The load of the systems was neutralized before simulation by adding the required chlorine ions.

Peptide Synthesis

The NRWC peptide with the sequence of NRWCFAGRR-NH2 was synthesized by Biomatik Company (Canada) using solid-phase technology. High-Performance Liquid Chromatography (HPLC) and mass spectroscopy were used in order to quantitatively and qualitatively confirm the desired peptide that indicated 97.76% purity. The peptide was stored at -20 °C until use.

MIC and MBC Assessment

MICs of peptides were determined using a standard serial microdilution method in Mueller-Hinton Broth (MHB) (Sigma, USA) for each bacterial strain according to the procedures outlined by the CLSI. Various concentrations of peptide solution (400, 200, 100, 50, 25, 12.5, 6.3, and 3.1 µg/ml) were added to microtitre plates in a final volume of 200 µl of MHB, including 2×10^5 colony-forming units. Plates were incubated at the appropriate temperature for 24 h and the absorbance was measured at 600 nm.

The lowest peptide concentration that inhibited bacterial growth was considered MIC. The MBC is defined as the least concentration of bactericidal activity where 99.9% of microorganisms are dead. To determine MBC, 50 µl of wells that did not have any bacterial growth was cultured on

Mueller-Hinton agar medium and incubated for a week. Each well that had prevented the total development and growth of bacteria was considered as MBC.[15]

Cells Toxicity

Hela cell line was cultivated in RPMI-1640 medium (Gibco, USA) containing 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), penicillin-streptomycin solution (100 IU/ml penicillin, 100 µg/ml streptomycin) (Sigma, USA), and was incubated at 37 ºC with 5% CO_2 and 70% humidity. The cells were harvested by trypsin-EDTA, and adjusted to 1×10^5 cell/ml, and 100 µl of prepared cell suspension was added to each well of a microtiter plate. The cells were then incubated at 37 °C with 5% CO_2 for 24 hours. The designed peptide was added in desirable concentrations (3.1-400 µg/ml) and the plates were incubated for further 24 and 48 hours. The supernatant of each well was discarded and wells were treated with 50 µl of 5 mg/ml MTT solution (Sigma, USA). After 4 hours of incubation, the solution in the wells was discarded and 100 µl DMSO was added to each well to dissolve the formazan crystals. The absorbance of wells was read by an ELISA plate reader at 570 nm wavelength.[16]

Hemolytic Activity Assay

The hemolytic activity of the peptide was evaluated on human erythrocytes. A 10% erythrocyte suspension in PBS was treated by 2-1000 µg/ml concentration of peptide and incubated at 37 °C for 15 minutes. Erythrocyte suspension treated by Triton X100 (0.1%) was used as a complete hemolysis (OD_C). The cells were then centrifuged and optical absorption of supernatants was measured in 415 nm wavelength and hemolysis percentage was calculated using the following formula[16]:

$$\text{Hemolytic activity} = (OD_{test} - OD_{blank} / OD_C - OD_{blank}) \times 100$$

Cell Selectivity/Therapeutic Index (TI)

The TI is often used as a parameter to represent the specificity of AMP that is experimentally calculated by the ratio of HC_{50} (the peptide concentration causing 50% hemolysis of red blood cells) to MIC. Actually, TI is a widely accepted parameter to represent the specificity of AMPs for prokaryotic versus eukaryotic cells. Large values in TI indicate greater antimicrobial specificity.

Nitric Oxide Production by J774 Macrophage Cell Line

In order to evaluate nitric oxide production induction, J774 cell line was cultured in 10^5 cell/ml density and treated with different concentrations of peptide (1-200 µg/ml) in 96 well plates. The plates were incubated for a different time ranging between 24, 48, and 72 h. After these times, the supernatant of cells were collected and were studied in terms of the amount of nitric oxide production using the Griess method.[17]

Statistical Analysis

All of the experiments were performed in triplicate and the mean of all data was calculated. Using non-parametric methods and Man Whitney test, the data were analyzed. Confidence limits level of 95% was considered for analysis and $p<0.05$ was considered as a significant difference.

Results

In Silico Study of Peptide

The synthesized peptide with $C_{50}H_{77}N_{21}O_{10}S_1$ molecular formula and 1164.36 molecular weight has theoretical pI equal to 11.70 and results of hydrophobicity and net charge of NRWCFAGRR peptide were -0.50 (H) and +3, respectively. Based on the PROB score, the designed peptide did not show any significant hemolytic activity and the Instability Index (II) was calculated to be 124.45, which means the peptide is a stable molecule. The level of the aliphatic index and grand average of hydropathy value of the peptide were shown to be 9.09 and -1.24, respectively (Table 1). Finally, the secondary and tertiary structure of the designed peptide showed a random coil (Figure 1).

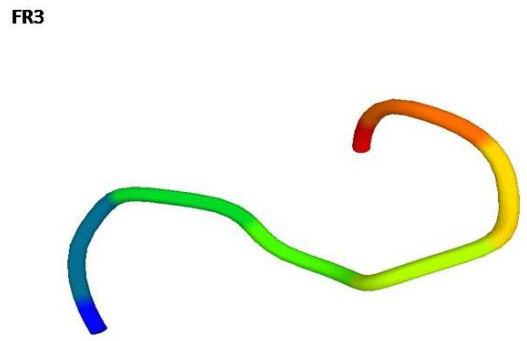

Figure 1. Tertiary Structure of NRWC Peptide.

Table 1. Result of *in Silico* Study of Peptide

PROB Score	Hydrophobic Residue %	Booman Index	II	TI	GRAVY	H	Aliphatic Index	Charge	pI	Mol wt.
0.49	44%	4.67	124.45	7.1	-1.24	-0.50	9.09	+3	11.70	1416.59

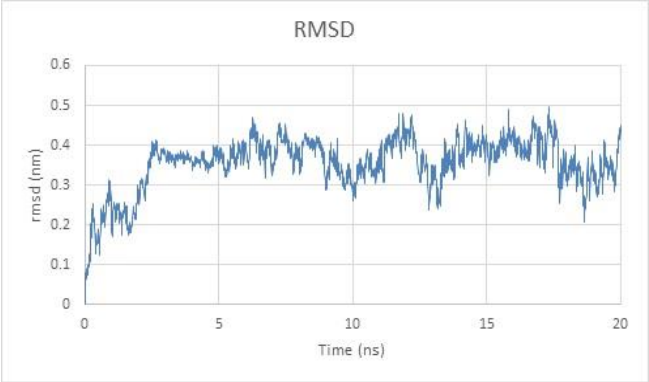

Figure 2. Root Mean Square Deviation (RMSD). The changes observed in rmsd and its increase indicate the existence of changes in the final structure with the original designed and modeled structure of the protein. Stabilization between rmsd changes in nanoseconds after 6 indicates a balance in the structure of the protein, and because the rate of change is less than 1 to 2 nm, the protein has not lost its overall modeled structure.

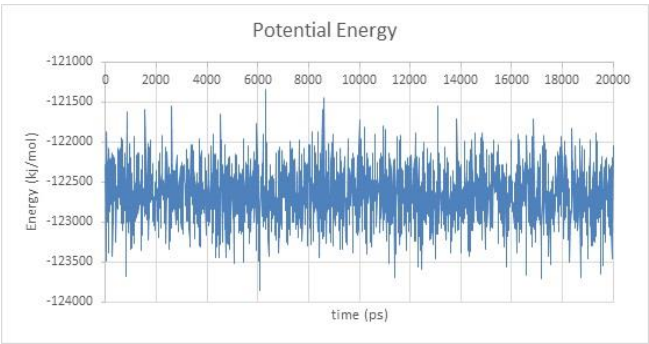

Figure 3. Potential Energy. Although changes in a specific 1000 kJ single interval indicate a large lack of change in protein structure, which in turn indicates that the molecule is not denatured.

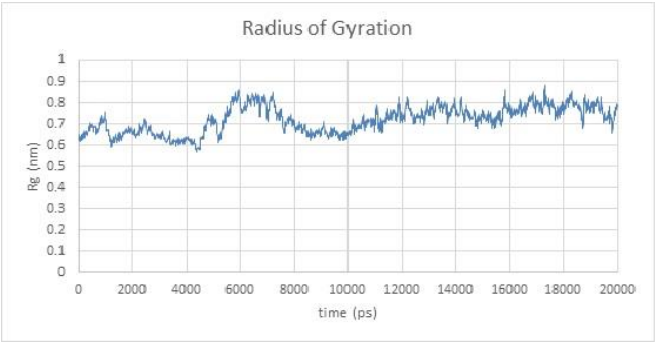

Figure 4. Radius of Gyration. Slight changes in the radius of gyration indicate no large-scale structure change in the three-dimensional structure of the protein and its spatial denaturation. Equilibrium in this characteristic indicates protein integrity and proper interaction with its environment.

After studying the proposed peptide structures from the Pep-fold and studying those structures' molecular dynamics, the results obtained from the graphs obtained from the MD peptide results were investigated.

Extensive structural changes have occurred at the beginning of the simulation and the initial modeled structure at the end of the simulation. Despite its stability, the protein does not have a consistent form and may not participate in intermolecular bonds. Such structural changes are due to the heterogeneity of amino acid structures along the peptide chain (Figure 2).

The lack of changes in the peptide potential energy indicates the peptide's overall structural stability and that the peptide is not denatured at all (Figure 3).

Changes in the radius of gyration in this peptide and the initial increase and final equilibrium process indicate changes in the peptide structure in three dimensions. The peptide exits its local minimum energy and returns structurally strongly, indicating initial wrong modeling. Despite many structural changes, it has not yet been fully denatured and has maintained its overall structure (Figure 4).

The multiplicative changes in the number of hydrogen bonds formed within the peptide molecule indicate a large change in the peptide structure. The initial structure and amino acid of this peptide are that amino acids capable of intramolecular bonding with R groups are spaced far apart. There is a large spatial barrier in this region so that the peptide's equilibrium. The number of these links is almost zero (Figure 5).

Extensive changes in pulsation in the number of available solvent levels indicate sudden structural changes abruptly at the peptide level. The peptide gradually leaves its simulated primary structure and interacts with the surrounding water molecules at each step, eventually reaching a stable state (Figure 6).

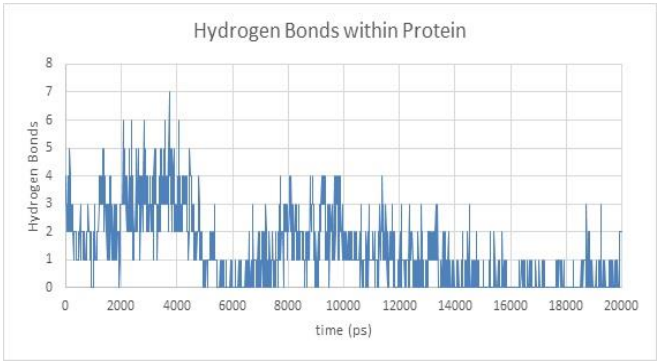

Figure 5. Hydrogen Bonds within Protein. The number of hydrogen bonds within a protein molecule indicates the number of intramolecular interactions, the reduction and equilibrium of which indicates the opening of the molecule and the lack of communication between the hydrogen bonds of the protein roots and the replacement of these bonds with water molecules. Represents the balance created in the molecule.

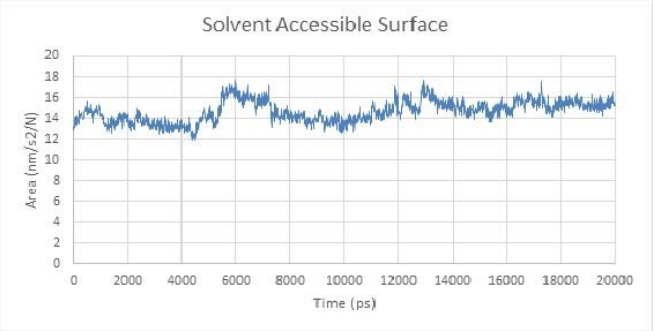

Figure 6. Solvent Accessible Surface the Available Solvent Level Refers to The Total Protein Levels That Are Associated with Water Molecules. The lack of large changes in this number indicates that the protein is not denatured and also maintains its modeled structure.

MBC and MIC

MIC and MBC of the designed peptide were investigated on 12 different bacterial strains. According to the results, the designed peptide has had an inhibitory effect on the growth of all 12 bacterial strains in various concentrations. However, these peptides had a killing effect only on *B. cereus*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* (Table 2).

Cell Toxicity

The cytotoxic effects of the peptide studied on Hela cell line using MTT assay was done after 24 and 48 h treatment. As shown in this test, this new peptide has no toxicity against mammalian cells in tested concentrations. This is due to the fact that in the MTT assay it was showed less than 5% toxicity in highest peptide concentration (400 µg/ml) and IC_{50} of this peptide is far from of these concentrations.

Hemolytic Analysis

Results of hemolysis evaluation showed that in 1 g/L concentration of NRWCFAGRR peptide, 1.2% of erythrocytes was lysed and in the antibacterial dose of peptide, there was no hemolytic activity. Because of the poor hemolysis activity of this peptide, HC_{50} and thereupon calculation of TI was impossible.

Table 2. Results of MBC and MIC Tests on 12 Bacterial Strains

Bacteria	*S. enteritis*	*B. cereus*	*E.coli*	*K. pneumonia*	*S. marcescens*	*A. baumannii*	*P. vulgaris*	*V. cholera*	*P. aeruginosa*	*S. aureus*	*S. epidermidis*	*N. brasiliensis*
MIC (µg/ml)	400	200	400	400	>400	400	>400	>400	400	200	400	400
MBC (µg/ml)	-	200	-	400	-	-	-	-	400	400	-	-

Nitric Oxide Production

Production of nitric oxide by J774 cell line treated with our designed peptide was also studied. After drawing the standard curve of nitric acid and using the resulting equation, the quantity of nitric oxide produced by macrophage cells treated with various concentrations of peptide 24, 48, and 72 hours after treatment was measured. The results revealed that the highest nitric oxide production level was observed 48 hours after culture of the antimicrobial peptide-treated cell line (Figure 2). The capability of this peptide has been demonstrated in the release of nitric oxide by the macrophage.

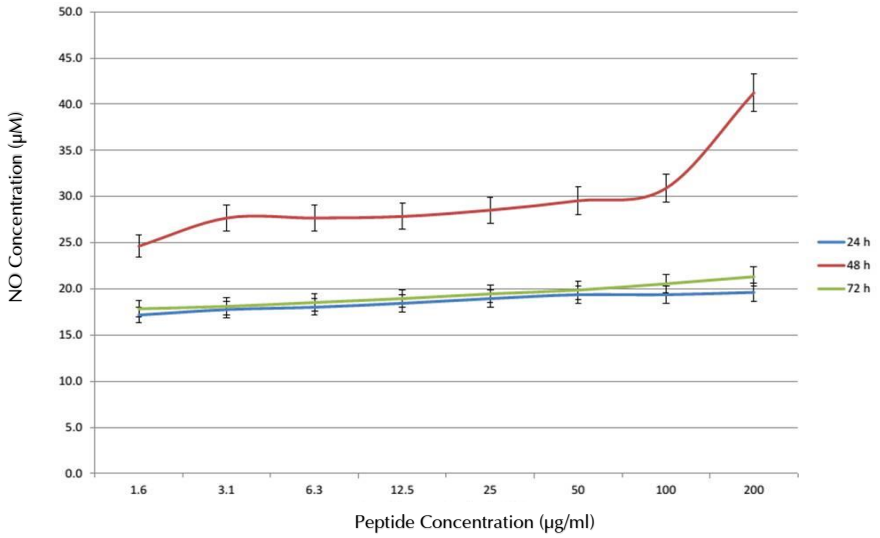

Figure 7. Nitric oxide Production by J774 Macrophage Cell Line Treated with Different Concentrations of Peptide within 24, 48, and 72 Hours.

Discussion

The current and past research on antimicrobial peptides have shown that these compounds have an excellent potential to be utilized in the medical and food industries. Constant discovery of new antimicrobial peptides and realizing their procedures and immune systems that play a major role in antimicrobial molecule synthesis, immunity and peptide configuration have paved the way for progress in this field with an emphasis on scientific application in the industry.[15,18]

Antimicrobial peptides are in fact natural substrates capable of connecting with a variety of enzymes and receptors. Thus, certain modifications are required to create appropriate pharmacological properties such as stability, specificity for a specific enzyme or receptor.[19]

In the present study, for the first time, the antibacterial effect of our designed peptide with NWRCFAGRR sequences has been determined against clinical important bacteria that usually show broad-spectrum resistance against antibiotics.

The results of MIC showed an antibacterial effect of peptide at a concentration of 200-400 and more than 400 µg/ml and results of MBC were in the same range of mentioned peptide. Among all the studied bacteria, the best MIC concentration of peptide was related to *S. aureus* and *B. cereus,* whereas the best result for MBC was related to *B. cereus.*

Zardini et al., in 2015 investigated the antibacterial and antifungal effect of Mastoparan-S as a new antibacterial peptide and showed higher antimicrobial activity against Gram-negative bacteria compared to Gram-positive ones. and fungi and the minimum inhibitory concentration (MIC) values of Mastoparan-S are 15.1–28.3 µg/ml for bacterial and 19.3–24.6 µg/ml for fungal pathogens.[20]

The comparison among the MIC of this new peptide and other peptides showed a higher MIC for this peptide compared to other peptides such as Temporin-Ra, Temporin-Rb, Buforin K, CA-MA, CA-MA1, CA-MA2, and CA-MA3.[21,22] Contrary to the similar study conducted by Teixeria et al., on the antibacterial effect of NWRCFAGDD peptide,[13] our peptide showed no cell toxicity in antibacterial concentration.

In 2013, Teixeria et al., studied the antimicrobial effect of NWRCFAGDD peptide on *Haemophilus parasuis.* A complete inhibition growth was observed at 20 µg/ml peptide on *H. parasuis* in this study. The results of their research indicated a strong antimicrobial effect on this bacterium.[13] In 2010, Rosenfeld et al., showed that addition of positive charge or peptide's hydrophobicity can enhance the antimicrobial potency of these peptides compared to the original peptide.[23] Considering the above experience and based upon the antimicrobial properties of NWRCFAGDD and the influence of positive charge on the antimicrobial activity of peptides, aspartate amino acid has been substituted with arginine residue to improve its antibacterial function.

However, the peptide studied in this research constituted a relatively low percentage (1.2%) of hemolysis on human erythrocytes. Whereas Moghaddam et al., in 2014 investigated the hemolytic effect of cationic CM11 peptide on human erythrocytes in MIC range and reported that at 64 µg/ml concentration of CM11 peptide, cytotoxicity on blood red cells was about 10%.[16] In another study, Duval et al., in 2009 demonstrated that at 160 µg/ml concentration of K4 cationic peptide hemolysis activity was 6.65%.[24] Zare-Zardini et al., investigated Mastoparan-S antibacterial peptideon human red blood cells and reported that only 3% of erythrocyte was lysed at 100 µg/ml concentration of peptide.[20]

The cytotoxic effect of our peptide did not show any considerable toxicity even in the highest concentration of peptide (400 µg/ml), whereas, in another study, Moghaddam et al., in a similar test with cationic CM11 peptide demonstrated no significant cytotoxicity on Hela cell line under 6 µg/ml after 24 and 48 h of treatment.[16]

Nitrite oxide is one of the most important products of macrophages that has an antimicrobial effect and helps the immune system to eradicate especially intracellular microorganisms.[25,26]

Therefore, the production of nitrite oxide as a crucial factor in j774 macrophage cell line is assessed for determining the indirect bactericidal potency of our candidate peptide.[27] The results of the present study shows the potency of the peptide in the stimulation of nitric oxide production by macrophage cell line. This valuable finding is important as it confirms that the candidate peptide is able to kill the microorganisms by two different mechanisms. In the direct mechanism, it can kill some microorganisms and in the indirect mechanism, it can potentially kill intracellular microorganisms by induction of nitric oxide production in the macrophages. Theoretically, by the induction of nitric oxide in the macrophages and nonspecific function of nitric oxide on microorganisms, this mechanism may be more effective in the *in vivo* condition and also more effective on more widespread organisms.

In the study of Scott et al., the antimicrobial effect of CEMA peptide on RAW264/7 macrophage cell line shows suppression of *iNOS* gene (nitric oxide synthesis induced by LPS) expression.[28] This finding shows that the peptide kills the organisms via direct mechanism and indirect mechanism in not involved for the killing of organisms by induction of nitric oxide. Hoyt et al., in 2005 investigated the effect of doxycycline on nitric oxide production in murine lung epithelial cells. They reported that doxycycline decreased nitric oxide production by the cells[29] while our designed peptide showed an antimicrobial effect in the *in vitro* assay and also induction of nitric oxide in macrophages.

Conclusion

Based on the studies conducted in the field of antimicrobial peptides, the peptide introduced in this research is a new

antimicrobial peptide. However, in this study its strong direct killing and antibacterial properties was not observed clearly. This is while the induction of nitric oxide by macrophages is an important finding that may be proposed that the *in vivo* activity of this peptide may be better than its *in vitro* antibacterial potency. *In vivo* studies are needed to determine its different biological effects and to prove our claim about the therapeutic potency of the studied peptide.

Funding

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Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

References

1. Fish DN. Optimal antimicrobial therapy for sepsis. Am J Health Syst Pharm. 2002;59(suppl_1):S13-9. doi:10.1093/ajhp/59.suppl_1.S13
2. Bastos P, Trindade F, da Costa J, Ferreira R, Vitorino R. Human antimicrobial peptides in bodily fluids: current knowledge and therapeutic perspectives in the postantibiotic era. Med Res Rev. 2018;38(1):101-46. doi:10.1002/med.21435
3. Moghaddam MM, Aghamollaei H, Kooshki H, Barjini KA, Mirnejad R, Choopani A. The development of antimicrobial peptides as an approach to prevention of antibiotic resistance. Rev Med Microbiol. 2015;26(3):98-110. doi:10.1097/MRM.0000000000000032
4. Mahlapuu M, Hekansson J, Ringstad L, Bjurn C. Antimicrobial peptides: an emerging category of therapeutic agents. Front Cell Infect Microbiol. 2016;6:194. doi:10.3389/fcimb.2016.00194
5. Struwe J. Fighting antibiotic resistance in Sweden–past, present and future. Wien Klin Wochenschr. 2008;120(9):268. doi10.1007/s00508-008-0977-6
6. Moravej H, Moravej Z, Yazdanparast M, Heiat M, Mirhosseini A, Moosazadeh Moghaddam M, et al. Antimicrobial peptides: features, action, and their resistance mechanisms in bacteria. Microb Drug Resist. 2018;24(6):747-67. doi:10.1089/mdr.2017.0392
7. Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?. Nat Rev Microbiol. 2005;3(3):238-50. doi:10.1038/nrmicro1098
8. Boulanger N, Bulet P, Lowenberger C. Antimicrobial peptides in the interactions between insects and flagellate parasites. Trends Parasitol. 2006;22(6):262-8. doi:10.1016/j.pt.2006.04.003
9. Moravej H, Fasihi-Ramandi M, Moghaddam MM, Mirnejad R. Cytotoxicity and antibacterial effect of Trp-substituted CM11 cationic peptide against drug-resistant isolates of *Brucella melitensis* alone and in combination with recommended antibiotics. Int J Pept Res Ther. 2019;25(1):235-45. doi:10.1007/s10989-017-9658-5
10. Chou HT, Wen HW, Kuo TY, Lin CC, Chen WJ. Interaction of cationic antimicrobial peptides with phospholipid vesicles and their antibacterial activity. Peptides. 2010;31(10):1811-20. doi:10.1016/j.peptides.2010. 06.021
11. Zasloff M. Innate immunity, antimicrobial peptides, and protection of the oral cavity. Lancet (London, England). 2002;360(9340):1116-7. doi:10.1016/S0140-6736(02)11239-6
12. Li L, Vorobyov I, Allen TW. The different interactions of lysine and arginine side chains with lipid membranes. J Physic Chem B. 2013;117(40):11906-20. doi:10.1021/jp405418y
13. Teixeira ML, Dalla Rosa A, Brandelli A. Characterization of an antimicrobial peptide produced by *Bacillus subtilis* subsp. *spizezinii* showing inhibitory activity towards *Haemophilus parasuis*. Microbiology. 2013;159(Pt_5):980-8. doi:10.1099/mic.0.062828-0
14. Fasihi-Ramandi M, Amani J, Salmanian AH, Moazzeni SM, Ahmadi K. In silico designing, cloning, and heterologous expression of novel chimeric human B lymphocyte CD20 extra loop. Tumor Biol. 2016;37(9):12547-53. doi:10.1007/s13277-016-5105-z
15. Moosazadeh Moghaddam M, Eftekhary M, Erfanimanesh S, Hashemi A, Fallah Omrani V, Farhadihosseinabadi B, et al. Comparison of the antibacterial effects of a short cationic peptide and 1% silver bioactive glass against extensively drug-resistant bacteria, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, isolated from burn patients. Amino Acids. 2018;50(11):1617-28. doi:10.1007/s00726-018-2638-z
16. Moghaddam MM, Barjini KA, Ramandi MF, Amani J. Investigation of the antibacterial activity of a short cationic peptide against multidrug-resistant *Klebsiella pneumoniae* and *Salmonella typhimurium* strains and its cytotoxicity on eukaryotic cells. World J Microbiol Biotechnol. 2014;30(5):1533-40. doi:10.1007/s11274-013-1575-y
17. Sun J, Zhang X, Broderick M, Fein H. Measurement of nitric oxide production in biological systems by using Griess reaction assay. Sensors. 2003;3(8):276-84. doi:10.3390/s30800276
18. Pometto A, Shetty K, Paliyath G, Levin RE, editors. Food biotechnology. CRC Press; 2005.
19. Hassan M, Kjos M, Nes IF, Diep DB, Lotfipour F. Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. J Appl Microbiol. 2012;113(4):723-36. doi:10.1111/j.1365-2672.2012.05338.x
20. Zare-Zardini H, Taheri-Kafrani A, Ordooei M, Ebrahimi L, Tolueinia B, Soleimanizadeh M. Identification and biochemical characterization of a new antibacterial and antifungal peptide derived from the insect *Sphodromantis viridis*. Biochemistry (Moscow). 2015;80(4):433-40. doi:10.1134/S0006297915040069
21. Asoodeh A, Zardini HZ, Chamani J. Identification and characterization of two novel antimicrobial peptides, temporin-Ra and temporin-Rb, from skin secretions of the marsh frog (*Rana ridibunda*). J Pept Sci. 2012;18(1):10-6. doi:10.1002/psc.1409
22. Shin SY, Kang JH, Jang SY, Kim Y, Kim KL, Hahm KS. Effects of the hinge region of cecropin A (1–8)–magainin 2 (1–12), a synthetic antimicrobial peptide, on liposomes, bacterial and tumor cells. Biochim Biophys Acta. 2000;1463(2):209-18. doi:10.1016/S0005-2736(99)00210-2
23. Rosenfeld Y, Lev N, Shai Y. Effect of the hydrophobicity to net positive charge ratio on antibacterial and anti-endotoxin activities of structurally similar antimicrobial peptides. Biochemistry. 2010;49(5):853-61. doi:10.1021/bi900724x
24. Duval E, Zatylny C, Laurencin M, Baudy-Floc'h M, Henry J. KKKKPLFGLFFGLF: a cationic peptide designed to exert antibacterial activity. Peptides. 2009;30(9):1608-12. doi:10.1016/j.peptides.2009.06.022
25. Bogdan C. Nitric oxide synthase in innate and adaptive immunity: an update. Trends Immunol. 2015;36(3):161-

78. doi:10.1016/j.it.2015.01.003
26. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. Annu Rev Immunol. 1997;15 (1):323-50. doi:10.1146/annurev.immunol.15.1. 323
27. Park E, Levis WR, Greig NH, Euisun J, Schuller-Levis G. Effect of thalidomide on nitric oxide production in lipopolysaccharide-activated RAW 264.7 cells. J Drugs Dermatol: JDD. 2010;9(4):330.
28. Scott MG, Rosenberger CM, Gold MR, Finlay BB, Hancock RE. An α-helical cationic antimicrobial peptide selectively modulates macrophage responses to lipopolysaccharide and directly alters macrophage gene expression. J Immunol. 2000;165(6):3358-65. doi:10.4049/jimmunol.165.6.3358
29. Hoyt JC, Ballering J, Numanami H, Hayden JM, Robbins RA. Doxycycline modulates nitric oxide production in murine lung epithelial cells. J Immunol. 2006;176(1): 567-72. doi:10.4049/jimmunol.176.1.567