Iran Red Crescent Med J. 2017 December; 19(12):e64178. doi: 10.5812/ircmj.64178.

Published online 2017 November 26. **Research Article**

Investigation of the Inhibitory Effects of the Designed NK95 Peptide on Expression of *SAP4-SAP6* Genes of *Candida albicans* in Comparison with Caspofungin

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Received 2016 October 03; **Revised** 2016 November 04; **Accepted** 2016 December 01.

Abstract

Background: *Candida albicans* is the most common pathogen in patients with vulvovaginitis. Secretory aspartyl proteinases (SAPs) are potential factors in the virulence of *C. albicans*. Antimicrobial peptides (AMPs) are recognized as a promising antimicrobial group of drugs with a membrane degradation mechanism. Occurrence of resistance to these drugs is quite rare. The impact of rapid membrane degradation on AMPs is relative to the prevention or delay in drug resistance mechanisms in different microbes.
Objectives: The purpose of this study was to compare the inhibitory effects of the designed NK95 peptide and caspofungin on expression of *SAP4-SAP6* genes from clinical vaginal isolates of *C. albicans* .
Methods: In this experimental study, samples were collected from 350 women, who were suspected of vulvovaginal candidiasis and were referred to Zahedan gynecology clinics during 6 months. The clinical specimens were cultured in Sabouraud glucose agar (SGA). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to identify the vaginal isolates of *C. albicans*. Afterwards, the NK95 peptide was designed and synthesized. Drug susceptibility test was carried out at different concentrations of NK95 peptide and caspofungin. RNA extraction, cDNA synthesis, and quantitative Real-time PCR were performed on the clinical isolates before and after treatment with the designed peptide and caspofungin.
Results: Based on the findings of this study, the minimum inhibitory concentration (MIC_{90}), MIC_{50}, and minimum fungicidal concentration (MFC) of *C. albicans* isolates were 62.5, 31.25, and 125 μg/mL for the designed peptide, respectively. The *SAP4* (45%), *SAP5* (85%), and *SAP6* (73%) genes expressions significantly reduced at 48 hours after treatment with the designed peptide ($P < 0.05$).
Conclusions: Our findings showed that the designed NK95 peptide has antifungal effects on the clinical vaginal isolates of *C. albicans*. Moreover, it has potential down-regulating on expression of SAP4, SAP5 and SAP6 genes.

Keywords: Antifungal Peptide, NK-95, SAPs, *Candida albicans*

1. Background

Vulvovaginal candidiasis is an important gynecological problem in medical practice. Almost 75% of women suffer from vaginal candidiasis at least once in their lifetime (1). In the past 3 decades, the incidence of invasive fungal infections has increased. In fact, female genital tract infections are among increasingly common fungal infections in clinics (2). *Candida albicans* is the most common pathogen causing vulvovaginitis. The virulence factors in *C. albicans* include fungal adhesion to mucosal surfaces, changing from yeast to hyphae form, mannan production, generation of secretory aspartyl proteinases (SAPs), as well as phospholipase and biofilm formation, which facilitate tissue invasion (3, 4). Overall, SAPs are of great importance in the development and pathogenesis of yeast infections.

SAPs are one of the key enzymes involved in the virulence of *C. albicans*. These enzymes lead to nonspecific proteolysis in host proteins involved in defense against infections; subsequently, *Candida* pass through the barriers of connective tissues (5). SAP is a hydrolytic enzyme, produced by at least 10 different genes. *SAP4-SAP6* mRNA was first detected in the formation of hyphae and was shown to play a major role in the development of disseminated candidiasis. *SAP4-SAP6* genes have a potential capacity to cause disorders, as blood and salivary pH is almost neutral and hyphae formation is important during tissue invasion (6).

SAP4 plays a specific role in the development of vaginitis (7). During acute infection in vaginitis, *SAP4* and *SAP5* genes are expressed. In a previous study, *SAP6* gene expression was confirmed during vaginal infection, using quantitative real time-polymerase chain reaction (qRT-PCR) (8). Since fungi are regarded as eukaryotes, the available antifungal drugs can have detrimental effects on the host tis-

sue cells. In addition, increased mortality of systemic candidiasis due to lack of early detection and treatment calls for the detection of *C. albicans* virulence mechanisms and necessitates the discovery of new antifungal agents (9-11).

Antimicrobial peptides (AMPs) are a group of promising antimicrobial drugs, associated with membrane cell degradation (12, 13). Rapid membrane degradation by AMPs is relative to their effects on the prevention or delay in drug resistance mechanisms of different microbes. These AMPs have broad-spectrum effects and exhibit selective activities against different organisms, including Gram-positive bacteria (eg, *Staphylococcus epidermidis* and *Staphylococcus aureus*) and Gram-negative bacteria (eg, *Escherchia coli*, *Pseudomonas aeruginosa*), and yeasts such as *C. albicans* (14).

There are various AMPs, which show acceptable performance against various pathogens in different organisms (12). Despite this diversity, AMPs also have common features. For instance, they are relatively short (< 100 amino acids) and have a positive charge of +2 to +9, which is because of the presence of amino acids, such as arginine, lysine, and histidine. They also have the capacity for compliance, as well as amphiphilic structures with separate hydrophobic and hydrophilic domains.

Due to the positive net charge of public AMPs, they are described as cationic AMPs (15). AMPs have useful properties, such as a broad-spectrum yield, selectivity against germs, and rare bacterial resistance; therefore, they are suitable antimicrobial agents. A large number of AMPs have been produced with either changes in natural AMPs or de novo peptide design guidelines. Today, synthesis of AMPs with changes in the sequence of natural AMPs, such as cecropin, melittin, meganine, endolicidine, or temporine, is a way to find appropriate peptides for therapeutic applications (15, 16). These changes include adding, deleting, or moving one or more amino acids and shortening the N-terminal, carboxyl, and connecting parts of natural peptides.

It should be noted that the built-in analog shows less toxicity compared to natural peptides, while it has greater antimicrobial activities (17). Due to the potential effect of *SAP* genes on the pathogenicity of *C. albicans*, besides the emerging drug resistance, different strategies have been employed to reduce *SAP* gene expression and treat vulvovaginal candidiasis (18).

2. Objectives

The purpose of this study was to explore the inhibitory effects of the designed NK95 peptide on expression of *SAP4-SAP6* genes from the clinical vaginal isolates of *C. albicans* in comparison with caspofungin.

3. Methods

In this experimental, cross sectional study, nonprobability sampling was applied among 350 women, who were suspected of vulvovaginal candidiasis and were referred to Zahedan gynecology clinics during 6 months (October 2012 - March 2013) with symptoms, such as irritation, itching, unpleasant odor, and pain during intercourse, associated with vaginal secretions.

The patients were identified by gynecologists and classified according to their symptoms and clinical criteria. The data were collected using questionnaires and clinical observations. Patients were graded (mild, moderate, and severe), and their demographic characteristics, such as age, sex, history of vulvovaginal disease, underlying diseases, antibiotic use, immunosuppressive therapy, recurrent diseases, and diabetes, were collected (19).

After recording the patients' information, vaginal samples were prepared by experts, and each sample was added to a sterile Falcon tube, containing 1 mL of sterile 0.9% NaCl. From each sample, direct smears were prepared with KOH 10% and examined under a microscope. For the genotyping test, some sections of the swab were removed from the vaginal discharge, added to a Sabouraud glucose agar (SGA) medium (65.5 g/L; Merck, Germany) with chloramphenicol (50 mg in 10 CC of alcohol), and incubated for 48 hours at 35°C.

After reaching the desirable growth of microorganisms, fresh yeasts from the culture medium were selected to obtain 30 clinical *C. albicans* isolates among the clinical samples. In this study, standard clinical isolates of *C. albicans* ATCC10231 were used, and the equipment was calibrated by the reference company before the experiments. All the experiments were performed by a single researcher, and variables were measured by the same researcher.

3.1. PCR-Restriction Fragment Length Polymorphism (RFLP) for Identification of Clinical Vaginal Isolates

For genotypic detection of yeast strains, DNA genome was extracted from fresh yeast culture, using phenol-chloroform-isoamyl alcohol. PCR was carried out using primer pairs, ITS1 (TCCGTAGGTGAACCTGCG) and ITS4 (CCTCCGCTTATTGATATGC), for amplification of genetic regions (ITS1-5.8 sr.DNA-ITS2). The sequence of the primers was extracted from the NCBI database (http://www.ncbi.nlm.nih.gov) and forward and reverse primers were designed using Gene Runner version 3.

The substances were used in PCR according to the master mix kit protocol (Thermo, Lithuania). Substances including H_2O, primer F, primer R, template DNA, and Master Mix were added to the microtube. To optimize the PCR conditions, a temperature gradient (55 - 60°C) was used, and

the most favorable temperature for DNA binding was obtained for reverse and forward primers. After electrophoresis of PCR products and ensuring their positivity, the MSP1 enzyme was used to differentiate the strains of *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*.

Following that, 10 μL of the PCR product was removed, and fragments were amplified in the CCGG region using 1.5 μL of enzyme buffer and 0.5 μL of MSP1 enzyme (Thermo, Lithuania). Then, 3 μL of distilled water was added and maintained at 37°C for 3 hours. Through electrophoresis of the digested product on Agarose gel, observation of DNA fragments under UV rays, and analysis of different RFLP patterns, various *Candida* strains were identified. Finally, in order to differentiate *C. albicans* from *C. dubliniensis*, the Mbo1 enzyme was used (20).

3.2. In Vitro Antifungal Activity

3.2.1. Antifungal Agents

A standard antifungal powder of caspofungin was purchased from Sigma Life Science (Sigma-Aldrich). Caspofungin was dissolved in sterilized 50% (v/v) dimethyl sulfoxide/distilled water (DMSO/DW) at a final concentration of 5 mg/mL. The NK95 peptide was dissolved in 0.05% DMSO at a final concentration of 10 mg/mL. The final concentrations were within the range of 0.125 - 64 μg/mL for caspofungin and 9.76 - 5000 μg/mL for the NK95 peptide; all the solutions were stored at -20°C.

3.2.2. Design of NK95 Peptide

The effective peptides were selected from the previous research and peptide bank. Evaluation of physicochemical properties was performed using DNAsis software and online ProtParam application. To evaluate the peptide structure, online software, GOR IV or PHDsec, was used for assessing the secondary structure; the tertiary structure was predicted using server I-TASSER. Ultimately, online Antibp3-Imtech and Split 4.0 server were used to design new peptides. The structure and characteristics of the new peptide were determined using the mentioned software. In addition, the designed peptides were compared with peptides in the peptide bank, using BLAST and antimicrobial databases.

3.2.3. Peptide Synthesis

The NK95 peptide was synthesized using the solid-phase method with pump A trifluoroacetic acid 0.1% in 100% water and pump B trifluoroacetic acid 0.1% in 100% acetonitrile (21). The crude peptide was repeatedly washed with diethyl ether, dried in vacuum, and purified using reversed-phase high-performance liquid chromatography (HPLC) on a 15-μm Deltapak C18 column (19 $\times$ 30 cm; Waters). The purity of the peptide was confirmed by analytical reversed-phase HPLC on an Ultrasphere C18 column (4.6 $\times$ 250 mm; Shimadzu). Purity of the peptide was above 80%. Electrospray ionization mass spectrometry was used to confirm the peptide identity.

3.3. Minimum Inhibitory Concentration (MIC) Determination

Broth microdilution assays were performed in triplicate according to the clinical and laboratory standards institute (CLSI) document M27-A3 (22). The final volume was about 200 μL in all the wells. All the plates were placed in a shaking incubator at 150 rpm for 48 hours at 37°C. Afterwards, turbidity of the wells was evaluated by examining the growth of yeasts. The lowest concentrations of NK95 peptide and caspofungin (without any turbidity) were considered as the MICs of the peptide and drug, respectively.

In order to determine the minimum fungicidal concentration (MFC), from MIC, and higher multidilution concentrations, culture studies were performed on Sabouraud-chloramphenicol agar. About 10 μL of the wells was transferred to the SGA medium and incubated at 37°C for 24 hours. Then, the number of colonies grown on SGA medium was measured, and MIC_{90}, MIC_{50}, and MFC of the designed peptide and caspofungin were obtained for the clinical isolates and *C. albicans* ATCC10231. Each experiment was carried out in triplicate and repeated at least 3 times.

3.4. Analysis of SAP4-SAP6 Genes Expression in Clinical Vaginal Isolates of C. albicans before and after Treatment with NK95 Peptide and Caspofungin

In order to compare the effects of NK95 peptide and caspofungin on *SAP4-SAP6* genes expression, vaginal *C. albicans* isolates were treated with the peptide and caspofungin at 24, 48, and 72 hours; the number of yeast cells for all the samples was determined at 1.5 $\times$ 10^3 CFU/mL. For the MIC_{50} concentration of the designed peptide and caspofungin, dilutions were separately prepared. In sterile 96-well plates, about 100 μL of the dilution, which was prepared separately for each isolate with 100 μL of yeast cells at 24, 48, and 72 hours, was treated; the plates were incubated at 37°C in a shaking incubator.

About 10 μL of the well content after treatment at 24, 48 and 72 hours was transferred to the SGA medium, containing chloramphenicol, and the plates were again incubated at 37°C. This process was included to obtain *C. albicans* colonie, treated with the NK95 peptide so that we could finally extract RNA from clinical isolates treated with the peptide at 24, 48, and 72 hours. To obtain *C. albicans* colonies treated with caspofungin at 24, 48, and 72 hours,

the same procedure was applied; each experiment was repeated at least 3 times.

3.4.1. RNA Isolation, cDNA Synthesis, and Quantitative Real-time PCR Assay

Total RNA was extracted using the CinnaClon RNA Isolation Kit (No., RN7713C), according to the manufacturer's instructions (CinnaClon, Karaj, Iran). For this purpose, 1 μg of total RNA was used as the template to synthesize cDNA. Reverse transcription was applied for 5 minutes at 25°C, for 1 hour at 42°C, and for 5 minutes at 70°C (thermo scientific RevertAid first strand cDNA synthesis Kit, Lot 00323337).

RNA extraction and cDNA synthesis were performed on the clinical vaginal isolates of *C. albicans* and standard strains of *C. albicans* ATCC10231 at various intervals before and after treatment with the NK95 peptide and caspofungin. To determine *SAP4-SAP6* genes expression, RT-PCR technique was used in the ABI StepOnePlus system (Applied Bio-Rad, USA). In this study, specific primers are presented in Table 1. The primers were synthesized by tag copenhagen (Denmark).

Table 1. Primer Sequences Used for the qRT-PCR

Gene	Primer Sequence	Amp Size (bp)
SAP4 (F)	5' AGA TAT TGA GCC CAC AGA AAT TCC3'	82
SAP4 (R)	5' CAA TTT AAC TGC AAC AGG TCC TCTT 3'	
SAP5 (F)	5' CAG AAT TTC CCG TCG ATG AGA3'	78
SAP5 (R)	5' CAT TGT GCA AAG TAA CTG CAA CAG3'	
SAP6 (F)	5'TTA CGC AAA AGG TAA CTT GTA TCA AGA3'	102
SAP6 (R)	5'CCT TTA TGA GCA CTA GTA GAC CAA ACG3'	
ACT1 (F)	5' CCA GCT TTC TAC GTT TCC 3'	209
ACT1 (R)	5' CTG TAA CCA CGT TCA GAC 3'	

The efficiency and melting temperature of primers were evaluated and approved by Gene Runner software. The materials required for RT-PCR were selected according to the protocols of SYBR Green high rox master mix kit (Ampliqon Company). For the RT-PCR analysis, each reaction mixture contained 12.5 μL of qPCR SYBR green master mix high ROX (1X), 1 μL of forward and reverse primers (0.1 pmol), and 1 μL of template (1 μg); finally, distilled water was added up to 25 μL in 96-well plates.

The RT-PCR reactions were performed at 95°C for 15 minutes, followed by 40 cycles for 5 minutes at 94°C, 30 seconds of annealing, and 30 seconds at 72°C. The annealing temperature was 50°C for *SAP4* and *SAP6* genes, 47°C for *ACT1* gene, and 53°C for *SAP5* gene in qRT-PCR. The results were analyzed using the $2^{-\Delta\Delta}$CT relative expression method (Livak method) (8). Each experiment was repeated at least 3 times.

3.5. Statistical Analysis

Statistical analyses were performed using IBM SPSS version 22. One-way analysis of variance (ANOVA) was performed with Tukey's method. P value below 0.05 was considered significant. All data are expressed as mean $\pm$ SD.

4. Results

4.1. Online Bioinformatic Investigation of the NK95 Peptide

Some research centers have developed AMP databases where substantial data about AMPs are collected and stored. Information, such as structural features of the desired sequence, hydrophobicity, cationic charge, peptides, and some other properties, is available to users. Table 2 indicates the properties of NK95 peptide (sequence, AYVRVRGVLVRYRRCWCVYAYVRVRGVLVR), using the following databases: dragon antimicrobial peptide database (DAMPD; www.apps.sanbi.ac.za/dampd/BioTools.php) by the bioinformatics research institute of South Africa, research institute of University of Nebraska, Eppley Institute, analytical design package (ADP2), and biomedical informatics centre of the national institute for research in reproductive health (NIRRH; Mumbai, India).

Table 2. The Results of Online Analysis of Eppley Institute, DAMPD, and NIRRH Databases

Variable	Value
AMP probability	0.990
Instability index	25.71
Aliphatic index	119.67
Hydropathy index	-0.29
Half-life	4.4 hours (mammalian reticulocytes in vitro). > 20 hours (yeasts in vivo) > 10 hours (*E. coli* in vivo)
Molecular weight	3687.4 g/mol
Boman index	-0.74 kcal/mol

Abbreviation: AMP, Antimicrobial peptide

4.2. Identification of Clinical Vaginal Isolates

Distribution of patients with acute and recurrent vaginitis in terms of predisposing factors is shown in Table 3. The PCR-RFLP findings showed that the size of fragments after enzyme digestion by the MSP1 enzyme in *Candida* strains was almost over 200 to 600 bp. The performance of MSP1 enzyme in RFLP products of all *C. albicans*

samples resulted in 2 distinct bands of 238 and 297 bp, respectively. Finally, 30 clinical isolates were confirmed as *C. albicans* and used for the following analyses.

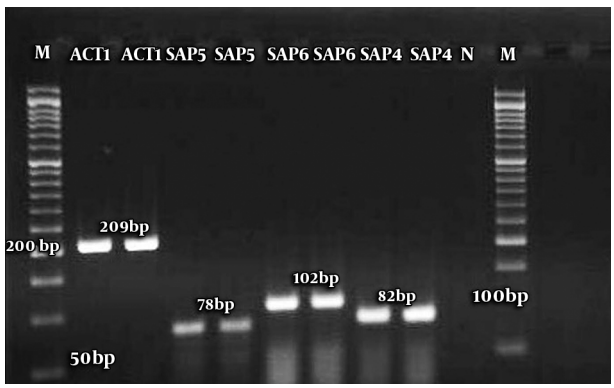
4.3. Determination of MICs

MICs was determined via broth microdilution method. To measure MIC in all the wells, cultures were examined on SGA, containing chloramphenicol. After counting the colonies, MIC_{50} values of 8 and 31.25 μg/mL were determined for caspofungin and NK95 peptide, respectively. The MIC_{90} and MFC of NK95 peptide and caspofungin are shown in Table 4.

4.4. RNA Extraction, cDNA Synthesis, and Quantification of SAP4, SAP5, SAP6, and ACT1 Gene Expression from Clinical Vaginal Isolates of C. albicans Before and After Treatment with NK95 Peptide and Caspofungin

Three bands, including 5srRNA, 18srRNA, and 28srRNA, were obtained in this method. To control the performance of primers, an appropriate PCR protocol was applied before qRT-PCR, and the construction of cDNA was verified. The RT-PCR reaction with the primers was separated after electrophoresis of PCR products on Agarose gel; the results indicated an exclusive product (Figure 1). The levels of *SAP4*, *SAP5*, and *SAP6* genes expression after treatment with NK95 peptide and caspofungin were examined by qRT-PCR at the obtained MIC concentrations.

Figure 1. The Electrophoresis Product of RT-PCR on *SAP4-SAP6* and *ACT1* Genes



M:DNA marker: 50bp; ACT1 gene bands: 209bp; SAP5 gene bands: 78bp; SAP6 gene bands: 102bp; SAP4 gene bands: 82bp; N:negative control.

The results of the proximity of vaginal *C. albicans* isolates to peptide NK95 showed a significant reduction in the expression level in all 3 genes. Gene expression of *SAP4* reduced by 33% at 24 hours, 45% at 48 hours after treatment, and 28% at 72 hours after treatment, compared to the samples which were not adjacent. *SAP5* gene expression reduced by 80% at 24 hours and 85% at 48 hours after the treatment (Table 5 and Figure 2).

5. Discussion

C. albicans is the most common pathogen involved in vulvovaginitis. One of the key enzymes in the virulence of *C. albicans* is SAP. In previous studies, it has been shown that *C. albicans* requires SAPs in the development of candidiasis; therefore, these enzymes can be a proper target for drug development (18, 23). AMPs are a good example, as laboratory studies have shown that the probability of resistance to AMPs is less than conventional antibiotics (24).

In the present study, the NK95 peptide (developed with respect to arenicin-1 peptide from *Arenicola marina*), was synthesized. With regard to the antimicrobial properties and biological activities discovered in the online software, the NK95 peptide was designed and synthesized, and the impact of its antifungal activity on the expression of *SAP4-SAP6* genes from clinical vaginal isolates of *C. albicans* was examined. The results of PCR-RFLP analysis of ITS1-5.8S-ITS2 fragment with the MSP1 enzyme (CCGG cutting site) showed 2 bands with different sizes in *C. albicans*, *C. dubliniensis*, *C. glabrata*, and *C. tropicalis* isolates, considering a cleavage site in the amplified fragment. Expectedly, this finding is consistent with the results of other studies.

In 2015, Diego and colleagues introduced PCR-RFLP as a valuable method for identification of invasive fungal infections (20). In other studies, Msp1 enzyme was used for segregation in *Candida* isolates (25, 26). In this study, the Msp1 enzyme was used to identify *C. albicans*, and 30 vaginal isolates of *C. albicans* were approved according to the band patterns. In the present study, the NK95 peptide was used to compare its effects on the expression of *SAP4-SAP6* genes with caspofungin as the reference drug. Although caspofungin could initially inhibit the synthesis of β1, 3-D-glucan in the cell wall, it showed an antifungal potential in delaying the expression of virulence factors, thus leading to infection caused by *C. albicans* (21).

Pfaller et al. performed a study in 2006 on the effect of antifungal caspofungin. In that study, a total of 8197 *Candida* isolates were collected from 91 medical centers during 2001 - 2004. Overall, 4454 (54%) cases were confirmed as *C. albicans*, and the MIC of caspofungin for the clinical isolates of *C. albicans* was 0.007-4 μg/mL, based on the broth microdilution method (25). Moreover, Mette and colleagues (2007) conducted a study on the effects of caspofungin on the standard T26 isolate of *C. albicans* and reported the MIC to be 8 μg/mL (28). According to the present results, the MIC_{90}, MIC_{50}, and MFC of caspofungin against the clinical isolates of *C. albicans* were 16, 8, and 32 μg/mL,

Table 3. Distribution of Patients with Acute and Recurrent Vaginitis in Terms of Predisposing Factors

Predisposing Factor	Recurrent Vaginitis		Acute Vaginitis	
	Frequency	Percentage (%)	Frequency	Percentage (%)
Underlying diseases	3	2.09	2	2.58
Antibiotics	84	58.71	27	34.83
Diabetes	19	13.28	15	19.35
Pregnancy	26	18.17	10	12.9
None	11	7.75	23	29.67
Total	143	100	77	100

Table 4. The MIC Ranges of NK95 Peptide and Caspofungin Against the Clinical Isolates of *C. albicans* and *C. albicans* ATCC10231

Antifungal	MIC Ranges for Antifungals (μg/mL)[a]					
	C. albicans ATCC10231			Clinical isolates of *C. albicans*		
	MIC_{50}	MIC_{90}	MFC	MIC_{50} range	MIC_{90} range	MFC
NK95 peptide	31.25	62.5	125	15.62 - 31.25	31.25 - 62.5	125
Caspofungin	8	16	32	4 - 8	8 - 16	32

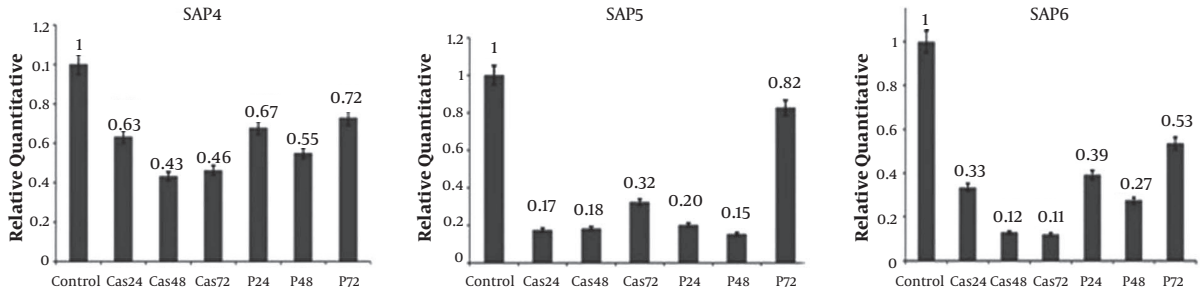
[a]The data in each column indicate 3 independent experiments ($P < 0.05$).

Table 5. *SAP4-SAP6* Gene Expression at 24 (A), 48 (B), and 72 (C) Hours of Treatment Induced by Culture Incubation at 37°C

A					
Gene	**Untreated *C. albicans***	**Caspofungin 4 μg/mL**		**NK95 Peptide 31.25 μg/mL**	
	Fold Change[a]	**Fold Change[a]**	**P Value[b]**	**Fold change[a]**	**P Value[b]**
SAP4	1	0.63	0.003	0.67	< 0.001
SAP5	1	0.17	< 0.001	0.20	0.019
SAP6	1	0.33	< 0.001	0.39	0.002
B					
Gene	**Untreated *C. albicans***	**Caspofungin 4 μg/mL**		**NK95 Peptide 31.25 μg/mL**	
	Fold Change[a]	**Fold Change[a]**	**P Value[b]**	**Fold Change[a]**	**P Value[b]**
SAP4	1	0.43	0.044	0.55	0.023
SAP5	1	0.18	0.012	0.15	< 0.001
SAP6	1	0.12	< 0.001	0.27	< 0.001
C					
Gene	**Untreated C. albicans**	**Caspofungin 4 μg/mL**		**NK95 peptide 31.25 μg/mL**	
	Fold change[a]	**Fold Change[a]**	**P Value[b]**	**Fold Change[a]**	**P Value[b]**
SAP4	1	0.46	0.025	0.72	< 0.001
SAP5	1	0.32	< 0.001	0.82	< 0.001
SAP6	1	0.11	0.016	0.53	0.045

[a]Fold change was calculated using the RT-PCR product of the gene of interest or PCR product of *ACT1* gene (housekeeping gene) and normalized to the negative control of untreated *C. albicans* (expression was considered to be 1).
[b]P values were obtained after comparison with the negative control (untreated *C. albicans*).

Figure 2. *SAP4*, *SAP5*, and *SAP6* Gene Expression After Treatment with Caspofungin (Cas) and NK95 Peptide (P) Compared to the Pretreatment



Control: sample before treatment; Cas24: 24 hours of treatment with caspofungin; Cas48: 48 hours of treatment with caspofungin; Cas72: 72 hours of treatment with caspofungin; P24: 24 hours of treatment with the peptide; P48: 48 hours of treatment with the peptide; P72: 72 hours of treatment with the peptide.

respectively; these findings are inconsistent with the results of previous studies in most cases.

In 2006, Ferre et al. examined the antimicrobial effects of CM11 peptide and 22 analogs on plant pathogens, *Erwinia amylovora*, *Xanthomonas vesicatoria*, and *Pseudomonas syringae*, and reported that the MIC of CM11 peptide for the pathogens was 7 - 10 mM (24). Moreover, Hong and colleagues (2015) designed antifungal peptides, which were more effective and safer than amphotericin B in the treatment of fungal keratitis in vivo, and the MIC of peptides (IKIK)2 and (IRIK)2 for *C. albicans* were 2, 31.3 mg/L and 3.9, 31.3 mg/L for *Fusarium solani*, respectively (29).

In another study, Cana and Dong (2009) designed an arenicin1 peptide, which could destroy phospholipid membranes. They also evaluated its antifungal activity against *C. albicans* and *non-C. albicans* species using the MTT assay. The MIC was 9 mM for *C. albicans* isolates and 4.5 to 9 mM for other isolates (30). Our results showed that the MIC_{90}, MIC_{50}, and MFC of the NK95 peptide were 62.5, 31.25 and 125 μg/mL for the clinical isolates of *C. albicans*, respectively, which are inconsistent with the MIC values reported in previous studies for plant pathogens and peptides (IKIK)2 and (IRIK)2 in the treatment of fungal keratitis due to *C. albicans*.

The observed difference in the inhibitory concentrations against different clinical isolates can be related to the peptide performance and membrane structure of the microbe. Researchers have suggested that the most important mechanism in the germicidal properties of most AMPs is to change the membrane structure. Accordingly, they associate the differences in susceptibility to variations in the plasma membranes of target microorganisms (ie, surface charge and lipid composition), which affect the extent and speed of interaction, cationic peptide binding to the plasma membrane surface, and power of peptides in plasma membrane disturbances (31). The findings of this

study showed that caspofungin and the designed peptide at concentrations of 8 and 31.25 μg/mL, respectively had significant differences in reducing *SAP4-SAP6* genes expression at 24, 48, and 72 hours after treatment, based on RT-PCR.

In this regard, Ripeau and colleagues in 2002 investigated the inhibitory effects of SAPs and phospholipase B1 of *C. albicans* in vitro. SAP and phospholipase B1 gene expression was assessed by RT-PCR after caspofungin was added to the cells and placed in SGA broth for 15 hours. SAP and phospholipase B1 gene expression was majorly inhibited in *C. albicans* after exposure to 4 - 16 μg/mL of caspofungin (21).

In addition, in another study in 2013, Theberge and colleagues examined the antifungal activity of decapeptide KSL-W against *C. albicans* ATCC-SC5314. The growth of *C. albicans* decreased with peptide KSL-W in short- and long-term incubation. Moreover, at a concentration of 10 μg/mL, the peptide effects were observed after 5 hours of incubation; however, the growth of *C. albicans* was completely inhibited at 25 μg/mL (32).

Overall, research activities are limited at the molecular level of the peptide (designed at the molecular level), even in the predesign of fungal virulence factors. In the present study, for the first time, the inhibitory effects of NK9 peptide on *SAP4-SAP6* gene expression in *C. albicans* were examined at the molecular level. As the results showed, 48 hours after treatment is the best time for the peptide to control *SAP4-SAP6* gene expression; similar results were reported for caspofungin.

The findings of this study showed that the effects of NK95 peptide in reducing *SAP5* gene expression were greater than caspofungin. In general, the designed peptide was more effective than caspofungin in reducing the expression of *SAP5* genes after 48 hours. Caspofungin exhibited poor performance in reducing *SAP6* gene expression at 24 hours after treatment and SAP5 gene expression at 24 and 48 hours after the treatment compared to the NK95 peptide.

Evaluation of the molecular inhibitory effects of NK95 peptide on *SAP4-SAP6* genes at 24, 48, and 72 hours of treatment, inclusion of a large number of clinical isolates, and evaluation of the concentration and physicochemical properties of NK95 peptide are among important and effective factors in the study of *SAP4-SAP6* gene expression, which differed from previous studies.

So far, treatment of invasive candidiasis has been effective with caspofungin. However, in recent reports, resistance to caspofungin has been reported in *Candida* species during treatment with esophagitis and endocarditis. Echinocandin resistance in *Candida* species is caused by a specific mutation in *FKS1* gene (encoding the necessary components for the synthesis of complex glucan) (33-35). Due to resistance to caspofungin, design of peptides can be an effective alternative to the treatment of vulvovaginitis caused by *C. albicans*.

5.1. Conclusion

Our findings suggested that the designed NK95 peptide had a potential application in the treatment and control of vulvovaginal disease caused by *C. albicans*. Also, the designed peptide had potential effects on decreasing SAP4 to 6 mRNA expression in *C. albicans*. Therefore, the designed peptide can be introduced as an appropriate alternative to azole drugs and an effective antifungal compound in preventing the pathogenicity of *C. albicans*.

Acknowledgments

This study was extracted from a PhD thesis, supported by Tarbiat Modares University, Tehran, Iran (1194506/94/02/20).

Footnotes

Authors' Contribution: Nasser Keikha obtained the specimens, performed all the tests, and wrote the draft of the manuscript; Mohammad Hossein Yadegari supervised the research; Masoumeh Rajabi Bazl designed and supervised the research and edited the draft; Jafar Amani designed the NK95 peptide and revised the draft of the manuscript; all authors reviewed and approved the final manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Moreira D, Paula CR. Vulvovaginal candidiasis. *Int J Gynaecol Obstet*. 2006;**92**(3):266–7. doi: 10.1016/j.ijgo.2005.12.007. [PubMed: 16434042].
2. Richter SS, Galask RP, Messer SA, Hollis RJ, Diekema DJ, Pfaller MA. Antifungal susceptibilities of Candida species causing vulvovaginitis and epidemiology of recurrent cases. *J Clin Microbiol*. 2005;**43**(5):2155–62. doi: 10.1128/JCM.43.5.2155-2162.2005. [PubMed: 15872235].
3. Hughes JA. *Candidiasis, Textbook of pediatric infectious disease*. Fifth , editor. Philadelphia: WB saunders company; 2004. p. 2569–79.
4. Hosseini SS, Yadegari MH, Rajabibazl M, Ghaemi EA. Inhibitory effects of carvacrol on the expression of secreted aspartyl proteinases 1-3 in fluconazole-resistant Candida albicans isolates. *Iran J Microbiol*. 2016;**8**(6):401–9.
5. Hube B, Monod M, Schofield DA, Brown AJP, Gow NAR. Expression of seven members of the gene family encoding secretory aspartyl proteinases in Candida albicans. *Mol Microbiol*. 1994;**14**(1):87–99. doi: 10.1111/j.1365-2958.1994.tb01269.x.
6. Edwards JE. *Candida species in mandell, douglas and bennett's principles and practice of infectious diseases*. 5 , editor. New York and London: Churchill Livingstone; 2000.

7. Taylor BN, Staib P, Binder A, Biesemeier A, Sehnal M, Rollinghoff M, et al. Profile of Candida albicans-secreted aspartic proteinase elicited during vaginal infection. *Infect Immun*. 2005;**73**(3):1828–35. doi: 10.1128/IAI.73.3.1828-1835.2005. [PubMed: 15731084].
8. Naglik JR, Rodgers CA, Shirlaw PJ, Dobbie JL, Fernandes-Naglik LL, Greenspan D, et al. Differential expression of Candida albicans secreted aspartyl proteinase and phospholipase B genes in humans correlates with active oral and vaginal infections. *J Infect Dis*. 2003;**188**(3):469–79. doi: 10.1086/376536. [PubMed: 12870130].
9. Scharbo-Dehaan M, Anderson DG. The CDC 2002 guidelines for the treatment of sexually transmitted diseases: implications for women's health care. *J Midwifery Womens Health*. 2003;**48**(2):96–104. [PubMed: 12686941].
10. Fidel PL, Cutright J, Steele C. Effects of reproductive hormones on experimental vaginal candidiasis. *Infect Immun*. 2000;**68**(2):651–7. doi: 10.1128/iai.68.2.651-657.2000.
11. Keikha N, Ayatollahi Mousavi SA, Nakhaei AR, Yadegari MH, Shahidi Bonjar GH, Amiri S. In Vitro Evaluation of Enzymatic and Antifungal Activities of Soil-Actinomycetes Isolates and Their Molecular Identification by PCR. *Jundishapur J Microbiol*. 2015;**8**(5):14874. doi: 10.5812/jjm.8(5)2015.14874. [PubMed: 26060560].
12. Hancock RE, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol*. 2006;**24**(12):1551–7. doi: 10.1038/nbt1267. [PubMed: 17160061].
13. Eckert R. Road to clinical efficacy: challenges and novel strategies for antimicrobial peptide development. *Future Microbiol*. 2011;**6**(6):635–51. doi: 10.2217/fmb.11.27. [PubMed: 21707311].
14. Ong ZY, Gao SJ, Yang YY. Short synthetic β-sheet forming peptide amphiphiles as broad spectrum antimicrobials with antibiofilm and endotoxin neutralizing capabilities. *Adv Funct Mater*. 2013;**23**(29):3682–92. doi: 10.1002/adfm.201202850.
15. Epand RM, Vogel HJ. Diversity of antimicrobial peptides and their mechanisms of action. *Biochim Biophys Acta*. 1999;**1462**(1):11–28. doi: 10.1016/s0005-2736(99)00198-4.
16. Montesinos E. Antimicrobial peptides and plant disease control. *FEMS Microbiol Lett*. 2007;**270**(1):1–11. doi: 10.1111/j.1574-6968.2007.00683.x. [PubMed: 17371298].
17. Ferre M R. *Design and synthesis of short antimicrobial peptides for plant protection, Study of their mode of action*. Spain: Universitat de Girona; 2010.
18. Kalkanci A, Bozdayi G, Biri A, Kustimur S. Distribution of secreted aspartyl proteinases using a polymerase chain reaction assay with SAP specific primers in Candida albicans isolates. *Folia Microbiol*. 2005;**50**(5):409–13. doi: 10.1007/bf02931422.
19. Fouladi B, Yadegari MH, Rajabibazl M, Fazaeli A, Hashemzadehchaleshtori M. Identification of candida species in patients with vulvovaginitis presenting different clinical symptoms. *J Zanjan Univ Med Sci Health Serv*. 2015;**23**(98):57–68. doi: 10.1007/bf02931422.
20. Sousa DRT, Santos CSD, Wanke B, Silva Júnior RMD, Santos MCD, Cruz KS, et al. PCR-RFLP as a useful tool for diagnosis of invasive mycoses in a healthcare facility in the North of Brazil. *Electron J Biotechnol*. 2015;**18**(3):231–5. doi: 10.1016/j.ejbt.2015.03.012.
21. Ripeau JS, Aumont F, Belhumeur P, Ostrosky-Zeichner L, Rex JH, de Repentigny L. Effect of the echinocandin caspofungin on expression of Candida albicans secretory aspartyl proteinases and phospholipase in vitro. *Antimicrob Agents Chemother*. 2002;**46**(9):3096–100. [PubMed: 12183282].
22. Clinical and Laboratory Standards Institute . *Reference method for broth dilution antifungal susceptibility testing of yeasts*. 3 , editor. Pennsylvania, Wayne: Clinical And Laboratory Standards Institute; 2008.
23. Cutler JE. Putative virulence factors of Candida albicans. *Annu Rev Microbiol*. 1991;**45**:187–218. doi: 10.1146/annurev.mi.45.100191.001155. [PubMed: 1741614].
24. Ferre R, Badosa E, Feliu L, Planas M, Montesinos E, Bardaji E. Inhibition of plant-pathogenic bacteria by short synthetic cecropin A-melittin hybrid peptides. *Appl Environ Microbiol*. 2006;**72**(5):3302–8. doi: 10.1128/AEM.72.5.3302-3308.2006. [PubMed: 16672470].
25. Farasat A, Ghahri M, Mirhendi H, Beiraghi S. Identification of candida species screened from catheter using patients with PCR-RFLP method. *Eur J Exerimen Biol*. 2012;**2**(3):651–6.
26. Mohammadi R, Mirhendi H, Yadegari MH, Shadzi S, Jalalizand N. Identification and frequency of candida species in patients with different forms of candidiasis in isfahan, using PCR-RFLP method. *J Isfahan Med Sch*. 2011;**2**(9):133.
27. Pfaller MA. Anidulafungin: an echinocandin antifungal. *Expert Opin Investig Drugs*. 2004;**13**(9):1183–97. doi: 10.1517/13543784.13.9.1183. [PubMed: 15330749].
28. Jacobsen MD, Whyte JA, Odds FC. Candida albicans and candida dubliniensis respond differently to echinocandin antifungal agents in vitro. *Antimicrob Agents Chemother*. 2007;**51**(5):1882–4. doi: 10.1128/aac.01525-06.
29. Wu H, Ong ZY, Liu S, Li Y, Wiradharma N, Yang YY, et al. Synthetic beta-sheet forming peptide amphiphiles for treatment of fungal keratitis. *Biomaterials*. 2015;**43**:44–9. doi: 10.1016/j.biomaterials.2014.11.052. [PubMed: 25591960].
30. Park C, Lee DG. Fungicidal effect of antimicrobial peptide arenicin-1. *Biochim Biophys Acta*. 2009;**1788**(9):1790–6. doi: 10.1016/j.bbamem.2009.06.008. [PubMed: 19539603].
31. Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol Rev*. 2003;**55**(1):27–55. doi: 10.1124/pr.55.1.2. [PubMed: 12615953].
32. Theberge S, Semlali A, Alamri A, Leung KP, Rouabhia M. C. albicans growth, transition, biofilm formation, and gene expression modulation by antimicrobial decapeptide KSL-W. *BMC Microbiol*. 2013;**13**:246. doi: 10.1186/1471-2180-13-246. [PubMed: 24195531].
33. Nevado J, De Alarcon A, Hernandez A. Caspofungin: a new therapeutic option for fungal endocarditis. *Clin Microbiol Infect*. 2005;**11**(3):248. doi: 10.1111/j.1469-0691.2005.01078.x. [PubMed: 15715728].
34. Mora-Duarte J, Betts R, Rotstein C, Colombo AL, Thompson-Moya L, Smietana J, et al. Comparison of caspofungin and amphotericin B for invasive candidiasis. *N Engl J Med*. 2002;**347**(25):2020–9. doi: 10.1056/NEJMoa021585. [PubMed: 12490683].
35. Kartsonis NA, Saah A, Lipka CJ, Taylor A, Sable CA. Second-line therapy with caspofungin for mucosal or invasive candidiasis: results from the caspofungin compassionate-use study. *J Antimicrob Chemother*. 2004;**53**(5):878–81. doi: 10.1093/jac/dkh179. [PubMed: 15044431].